Heliyon 10 (2024) e38932

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

# New horizon of the combined BCG vaccine with probiotic and liraglutide in augmenting beta cell survival *via* suppression of TXNIP/NLRP3 pyroptosis signaling in Streptozocin–Induced diabetes mellitestype-1 in rats

Amira Karam Khalifa [a,b,*], Dina Sayed Abdelrahim [c,d], Dina Mohamed Mekawy [e,f], Reham Mohammad Raafat Hamed [g], Wafaa Rabee Mohamed [h,i], Nagwa Mahmoud Ramadan [j], Mostafa Wael [k], Rawan Ellackany [k], Emad Ali Albadawi [l], Walla'a A. Osman [a]

[a] Department of Medical Pharmacology, Faculty of Medicine, Cairo University, El- Manial, Cairo 11562, Egypt
[b] Department of Medical Pharmacology, Faculty of Medicine, Nahda University, 62521, Beni Suef, Egypt
[c] Department of Clinical Pharmacology, Faculty of Medicine, Ain Shams University, Egypt
[d] Department of Pharmacology, Faculty of Medicine, Modern University for Technology and Information, Cairo, Egypt
[e] Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Badr University in Cairo, Badr City, Egypt
[f] Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt
[g] Department of Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University, Egypt
[h] Department of Histology and Cell Biology, Faculty of Medicine, Ain Shams University, Egypt
[i] Department of Histology and Cell Biology, Faculty of Medicine, Modern University for Technology and Information, Egypt
[j] Department of Physiology, Faculty of Medicine, Cairo University, El Manial, Cairo 11562, Egypt
[k] Faculty of Medicine, Modern University for Technology and Information, Cairo, Egypt
[l] Department of Basic Medical Science, College of Medicine, Taibah University, KSA, Saudi Arabia

ARTICLE INFO

*Keywords:*
Diabetes
TXNIP
NLRP3
Pyroptosis
Liraglutide
Probiotic
BCG
STZ

ABSTRACT

*Background:* An ideal anti-diabetic type-1 pharmacotherapy should combine abrogation of beta cell pyroptosis with enhancement of beta cell mass.
*Objectives:* The study investigated the potential synergism from combining the Bacillus Calmette-Guerin (BCG) vaccine with liraglutide (LIR) and probiotics in mitigating Streptozocin (STZ)-induced Type1diabetes mellitus in albino rats *via* suppression of TXNIP/NLRP3 signaling.
**Methods:** Induction of diabetes was performed by two I.V. injections of 50 mg/kg of STZ in male Wistar rats. Forty-eight rats were randomly allocated into six groups: Normal control group; STZ -diabetic group; BCG group; BCG + LIR group; BCG + probiotic group; BCG + LIR + probiotic group. The rats were sacrificed after 8 weeks of treatment.
*Results:* The STZ-diabetic group exhibited significant elevation of fasting blood sugar and HbA1c with remarkably decreased serum insulin along with a considerable increase in pancreatic proinflammatory cytokines (TNF-α, NLRP3, IL-1β, and NFκB) and apoptotic markers (ASK-1, IAPP, TXNIP, and Caspase-3) with prominently compromised oxidative scavenging capacity in

* Corresponding author. Faculty of Medicine, Cairo University, El Manial, Cairo, 11562, Egypt.
*E-mail addresses:* amirakaramkhalifa@cu.edu.eg, amira.karam@nub.edu.eg (A.K. Khalifa), dinapharma2008@med.asu.edu.eg (D.S. Abdelrahim), dinaa_dr@cu.edu.eg (D.M. Mekawy), rehamraafat1986@cu.edu.eg (R.M.R. Hamed), wafaa.rabee@med.asu.edu.eg (W.R. Mohamed), Dr-albadawi@hotmail.com (E.A. Albadawi), wala2osman@cu.edu.eg (W.A. Osman).

https://doi.org/10.1016/j.heliyon.2024.e38932
Received 2 November 2023; Received in revised form 1 October 2024; Accepted 2 October 2024
Available online 3 October 2024

addition to structural alteration in the pancreatic histoarchitecture with decreased insulin immunostaining. Conversely, diabetic-treated groups, especially the BCG + LIR + probiotic group, were superior in amelioration of STZ-induced pyroptosis of pancreatic islets evidenced by a significant decline in inflammatory cytokines and apoptotic markers with a remarkable upgrade in redox balance, Furthermore, the mitigation in the altered histopathological picture of the pancreas with enhanced insulin immunostaining has been was mirrored on the significant improvement of glucose homeostasis parameters.

*Conclusions:* Noteworthy, BCG combination with liraglutide and probiotic might be a promising repurposed therapeutic modality in the management of type-1 diabetes mellites *via* targeting pancreatic TXNIP/NLRP3 signaling pathway.

## 1. Introduction

The epidemic pattern of diabetes mellitus disease is prominently growing at an alarming rate and it is expected to reach 13·5 to 17·4 million in 2040 [1]. Diabetes mellitus Type 1 (T1DM) is an autoimmune illness mediated mainly by T lymphocytes, that leads to damage in insulin-secreting β-cells of islets of Langerhans, where the percentage of non-functioning pancreatic cells increases to a critical mass greater than 75 % that leads to subsequent absolute insulin deficiency which characterizes this type of diabetes [2].

It is well-known that individuals with type 1 diabetes must take insulin daily for the rest of their lives. Unfortunately, the currently available insulin replacement therapy does not cure T1DM but works by controlling hyperglycemia to prevent various complications in different systems of the body [3]. Additionally, even though β-cell death is involved in the development and progression of diabetes. However, little is known about boosting the survival of pancreatic β-cells. Thus, investigating novel approaches for the management of TIDM and associated complications is imperative.

There is a growing body of evidence that the primary pathogenic agents in the natural course of T1DM are the activated inflammatory pathways with downstream up-leveling of interlukin1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) [4]. Diabetes-related hyperglycemia was found to add to the chronic inflammation that lasts a long time and causes pathological alterations in the pancreatic β cells with subsequent generation of reactive oxygen species (ROS) [5]. Notably, pancreatic β-cells are more susceptible to oxidative stress due to their low expression level of antioxidant enzymes in comparison to other cell types in the pancreas leading to redox imbalance and apoptosis in pancreatic beta cells [6].

Thioredoxin-interacting protein (TXNIP) has been identified as a major contributor to β cell apoptosis. Of note, the redox imbalance with subsequent mitochondrial oxidative stress triggers the activation of TXNIP which functions as a negative regulator of thioredoxin (TXN2), an antioxidant that controls the cellular redox balance in β cells [7,8]. Various studies have emphasized that TXNIP is a critical link between hyperglycemia-related β cells toxicity and apoptosis of these cells [9–11].

Pyroptosis is a form of programmed cell death induced by the Nucleotide-binding domain, Leucine-Rich–containing family, Pyrin domain–containing-3 (NLRP3) inflammasomes [12]. Recently, Carlos et al. have hypothesized that NLRP3 inflammasomes have been implicated in the pathogenesis of diabetes mellitus and its consequences by eliciting sterile inflammatory responses [13]. Interestingly, Zhou et al. [14] have demonstrated that TXNIP is a key player in modulating NLRP3 inflammasomes activation. Accordingly, Targeting the TXNIP/NLRP3 signaling could be a promising approach for the management of T1DM.

Bacillus Calmette–Guérin vaccine was found to have a non-specific effect on the immune system, raising the possibility that it may have off-target therapeutic effects for humans, including the prevention or treatment of autoimmune illnesses like T1DM [15]. (BCG) vaccine is one of the oldest immunotherapies in clinical practice. Although several studies have evaluated the effect of BCG vaccination in T1DM and its role in modulating cellular immune mechanisms, however, the results are scarce and controversial.

The increased interest in the study of microbiome of the gut has paved the way for the use of probiotics in treating different types of diabetes such as *Lactobacilli* strains and *Bifidobacterium lactis* [16]. It is worth mentioning that similar to the gut, the pancreas has its own microbiota as well, and changes to this "pancreatic" microbiota are linked to intra-pancreatic immune responses and the development of diseases such as T1DM and pancreatic cancer [17]. More interestingly, recent studies revealed the positive correlation between dysbiome and NLRP3 inflammasomes activation with subsequent triggering of chronic inflammation that results in many various chronic metabolic diseases like diabetes mellites [18].

On the other hand, Glucagon-like peptide-1 (GLP-1) receptor agonists, have been proposed as a treatment to restore insulin secretion in recently diagnosed T1DM [19]. Interestingly, (GLP-1) agonists have been shown to downregulate the expression of TXNIP [20]. These findings have paved the way to consider TXNIP as a new promising therapeutic target. Consequently, searching for effective TXNIP inhibitors is currently a great pressing need for the treatment of DM. Interestingly, a recent study has speculated the potential effect of liraglutide to suppress cardiomyocyte pyroptosis *via* NLRP3 inflammasome suppression [21], raising our hypothesis that liraglutide might also target the pancreatic TXNIP/NLRP3 signaling pathway.

In view of these insights, this study has been designed to evaluate the possible synergistic antidiabetic action that can be attained from the combination of liraglutide and/or probiotic with BCG vaccine in streptozotocin-induced type1 diabetes mellites and elucidate the possible impact of these agents on TXNIP/NLRP3/IL1β pathway which could have a promising potential hope for diabetic patients.

## 2. .Materials and methods

### 2.1. sample size calculation

The sample size was calculated by the G*Power program (Version 3.1.9.2, by Franz Faul, Kiel, Germany) and adjusted appropriately according to a previous study by Ao et al. [22]. We supposed that our primary outcome is the level of pancreatic TXNIP expression and attrition rate of 20 %. According to that proposal, the sample size of 8 rats per group (4 rats for biochemical-related parameters and the remaining 4 rats for the pathological-related parameters). Our effect size was noticed with 95 % power and an alpha level of 5 % using a one-way ANOVA test within the six groups.

### 2.2. Animals and ethical statement

The study included 48 male, healthy, Wister albino rats matched for age (12 weeks) and weight (200–220) g. Rats were purchased and bred in the animal house of Kasralainy Faculty of Medicine, Cairo University, Egypt. Animals were kept under standardized conditions (Temperature 25 + 2 °C,12:12 light-dark cycle, and 50 + 5 % relative humidity) in plastic cage (33 cm × 40 cm × 17 cm). The rats were identified with a cage card indicating drug dose, group, and animal number. The investigational protocol was revised and approved by the Institutional Animal Care and Use Committee, Cairo University (CU-IACUC) (Approval number: CU-III/F/85-22) in accordance with ARRIVE guidelines**.** Rats had unrestricted access to chow pellets and water *ad libitum.* Rat tail venous samples were collected from the purchased animal to ensure their euglycemic state.

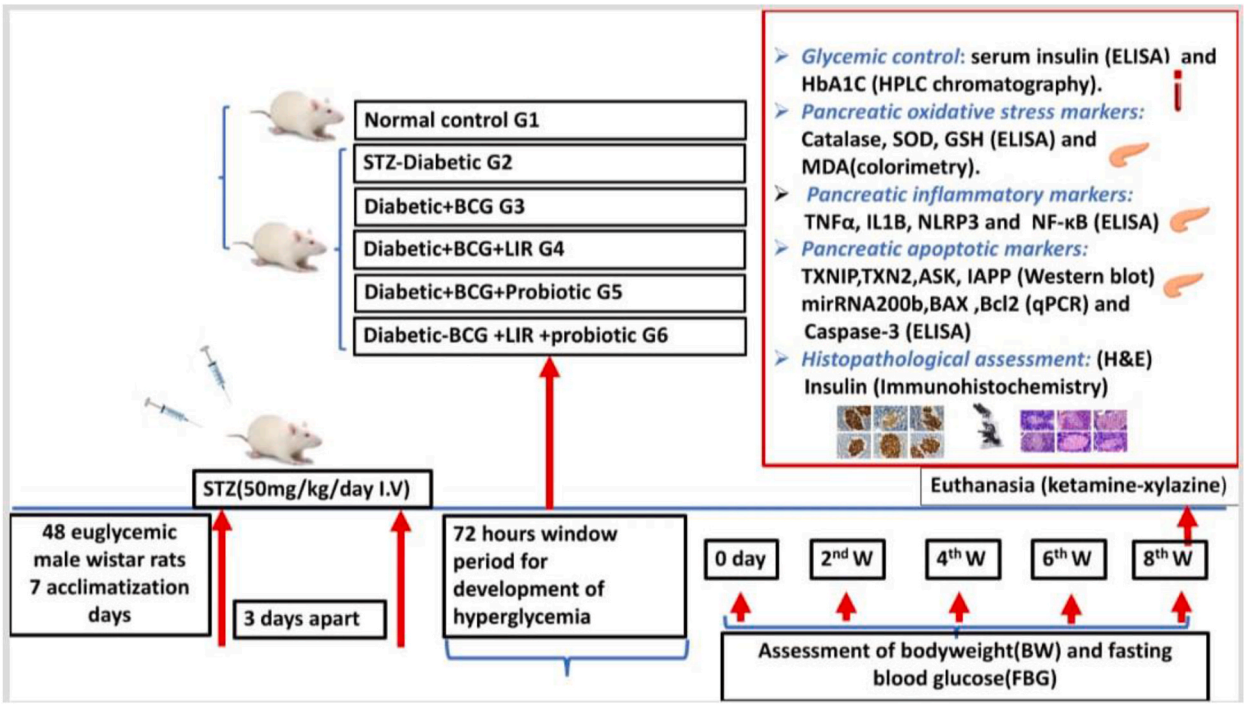
### 2.3. Drugs

**(STZ)**: Streptozotocin was purchased from Sigma Aldrich, Egypt (CAS No. 18883-66-4, Sigma No. S0130), 50 mg of Streptozotocin was dissolved in 0.01 M sodium citrate buffer (pH 4.5) and freshly used within 5 min according to the manufacturer's instructions. The final concentration was 50 mg/kg (2 ml/kg).

**BCG**: BCG live-attenuated strain purchased from VACSERA, Agouza, Giza, Egypt. Lyophilized powder diluted in 1 ml sterile saline solution, each 0.1 ml contains between $1.5x10^8$ colony forming unit (CFU) and stored under refrigerated conditions (2–8 °C).

**Probiotic**: Linex® Each capsule contains at least $1 \times 10^9$ CFU of *Lactobacillus acidophilus* and $1 \times 10^9$ CFU of *Bifidobacterium animal.* Each capsule content was dissolved in 1 ml phosphate buffer saline.

**Liraglutide**: (Victoza®) was obtained from Novo Nordisk S.p.A. (Rome, Italy) as pre-filled pens containing 18 mg LIR in 3 ml solution; 250 μl of this solution was diluted in 5 ml of saline solution (0.9 % NaCl) to obtain a final solution of 300 μg/ml.




**Scheme 1.** Timeline of the experimental workflow.

### 2.4. Experimental design and animal model establishment

The experimental timeline for the study is depicted in Scheme 1(supplementary file1). After 1week of acclimatization, induction of diabetes was performed by two I.V. injections of 50 mg/kg of STZ with 3 days apart in 6–8 h overnight fasted rats. The dose of STZ is selected according to studies done by Wang et al. [23] and Sariyanti et al. [24]. Rats had unrestricted access to food and 10 % sucrose water soon after the injection of STZ for 24h to prevent early hypoglycemia-related mortality, then shifted to regular water [25]. 72 h after the second STZ injection, the diabetic status of each animal was confirmed *via* measuring blood glucose levels from a tail snip after overnight fasting using a portable blood glucose meter (AccuCheck; Roche Diagnostics, Germany). All venous blood collection occurred under brief isoflurane anesthesia to prevent the animal restrained. Only animals that developed fasting blood glucose (FBG) level of more than 200 mg/dl (11 mmol) were included in our study and assigned for the treated groups [26]. Then 8 rats per group (each rat has a tag number) were allocated in six groups as follow:

**Group (1)**: Normal rats received I.V equivalent volume of citrate buffer once,0.1 ml S.C normal saline once at day 1 in addition to daily 1 ml PBS and throughout the experimental period and nominated as normal control; **Group (2)**: STZ -diabetic group. This group was nominated as a model non-treated group; **Group (3)**: Rats received a single dose of 0.1 ml S.C BCG vaccine and were nominated as the diabetic-BCG treated group; **Group (4)**: Rats received the forementioned dose of BCG in addition to liraglutide at a dose of 300 μg/kg daily subcutaneously [27] for 2 month and nominated as diabetic-BCG + LIR treated group; **Group (5)**: Rats received the forementioned dose of BCG in addition to 1 ml of probiotic crushed capsule ($1 \times 10^9$ CFU of Lactobacillus acidophilus CFU and $1 \times 10^9$ CFU of Bifidobacterium animalis) on empty stomach once a day in a dose of 1 ml by oral gavage for 2 month and nominated as diabetic-BCG + probiotic treated group; **Group (6)**: Rats received the triple combination of BCG, liraglutide and probiotic with the forementioned doses and nominated as diabetic-BCG + LIR + probiotic treated group. Body weight and fasting blood glucose levels were measured before and after STZ administration, as well as during drug treatment at an interval of 2 weeks by portable glucometer. After the end of the experimental period (8 weeks), rats of all studied groups were fasted overnight then rats were euthanized by 90 mg/kg ketamine–10 mg/kg xylazine cocktail, followed by terminal exsanguination. Blood samples (10–15 ml) were collected *via* cardiac puncture from the rats. Part of the blood samples were then centrifuged at 4000 g for 15 min, and serum was stored at −80 °C, the other part was collected in clean, dry tubes containing EDTA as an anticoagulant for the assessment of glycosylated hemoglobin. Afterward, the pancreas was excised and rinsed with ice-cold saline. One subset of pancreatic tissues was dissected and collected in liquid nitrogen and stored at −80 °C for Western blot analysis, ELIZA analysis, and gene expression. The other subset of pancreatic tissue was fixed in 10 % phosphate-buffered formalin, processed, embedded in paraffin, and cut into sections for hematoxylin and eosin staining and immunohistochemistry analysis.

### 2.5. Assessment of body weight change and mortality among the different studied groups

Mortality was assessed throughout the experimental period. Rats' body weight from the different studied groups was measured every 2 weeks to assess the change in their overall weight.

### 2.6. Blood and pancreatic tissue sampling

#### 2.6.1. Assessment of fasting blood glucose, serum insulin, and glycosylated hemoglobin (HbA1C)

The fasting blood glucose (FBG) was checked every 2 weeks in all experimental rats after overnight fasting from rat tail snip by portable glucometer. The levels of serum insulin were determined by the Enzyme-Linked Immunosorbent Assay kit specific for rat insulin (Ray Biotech., Norcross, Georgia, USA). 100 μl of serum sample was pipetted to each well then 100 μl of biotinylated antibody was added to each well and left for 1 h at room temperature with gentle shaking. Afterward,100 μl of streptavidin solution was added to each well and incubated for 45 min then 100 μl of TMB-One-Step Substrate Reagent to each well was added to each well for 30 min in the dark. Finally, the reaction was stopped 15 min later by adding 50 μl of stop solution to each well. The optical density was read at 450 nm. The read values were plotted on sigma plot software from which the values for the samples were extrapolated and individual blood samples from each group were used for (HbA1C) using a Glycohemoglobin (HbA1c) analyzer that utilizes High-Performance Liquid Chromatography.

#### 2.6.2. Assessment of the oxidative stress-related enzymes and proinflammatory cytokines

Following homogenizing the pancreatic samples, the supernatants were collected and kept at −80C after a 15-min centrifugation at 3000*g* and 4C. Then, using commercially available colorimetric detection kits (Bio-Diagnostic company, Giza, Egypt), according to the manufacturer's instructions, estimate the levels of oxidative stress markers in tissue supernatants to assess reduced glutathione (GSH), malonaldehyde (MDA) as a marker for lipid peroxidation, superoxide dismutase (SOD), and Catalase (CAT) levels. GSH, MDA, SOD, and CAT were quantified by measuring absorbance at 405 nm, 534 nm, 560 nm and 500 nm, respectively. Additionally, proinflammatory cytokines, including TNF-α, IL-1β, NF-κB, and NLRP3 were evaluated using commercial ELISA kits. Rat TNF-α(E-EL-R0019 Elabascience Biotechnology INC ,Hoston,Texax,USA).Rat IL-1β ELISA Kit(E-EL-R0012 Fine Biotech Co., Ltd, Wuhan, China, Rat NLRP3 (ER1965 Fine Biotech Co., Ltd, Wuhan, China), Rat pS536-NFκB-p65(MBS9511033, My BioSource, CA, USA. ELISA readers were used to measure the color absorbance of the ELISA kits in the 490 to 630 nm OD range (Florida, USA).

*2.6.3. Assessment of pancreatic expression of apoptotic markers*

*2.6.3.1. Assessment of pancreatic content of Caspase-3 by enzyme-linked immunosorbent assay.* Rat Caspase-3(CSB-E08857r, CUSBIO BIOTEC CO., Wuhan, China).Caspase-3 values of the different studied groups were extrapolated by plotting the log concentration of caspase-3 versus the log of the O.D.

*2.6.3.2. Assessment of TXNIP, $TXN_2$, ASK-1, and IAPP relative expressions by Western blot analysis.* Pancreatic tissues were homogenized in a phosphatase and protease inhibitor cocktail-supported RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, and 0.1 % SDS) to preserve protein integrity. Following protein analysis, 10 g of each sample's proteins were loaded, separated by SDS-PAGE, transferred to PVDF membranes, and blocked with 5 % bovine serum albumin (BSA). The membrane was then incubated with anti-TXNIP (1:1000; cat#: GTX57221, GeneTex, Inc., North America), anti-TXN2 (1:1000; cat#: 13089-1-AP, Protientech, USA), anti-ASK-1(phosphorThr838) (1:1000; cat#:28201-AP, Protientech, USA), or anti-IAPP (1:1000; cat#: TA321122, OriGen Technologies, Inc. Finally, the protein was assessed using densitometric analysis and a scanning laser densitometer after the blots were seen with enhanced chemiluminescence detection reagents (Amersham Biosciences, NJ, USA) (GS-800 system, Bio-Rad, CA, USA). The results are presented as arbitrary units (AU) after normalization for β-actin housekeeping protein expression for ASK and IAPP. Normalization for glyceraldehyde -3-phosphate dehydrogenase (GAPDH) housekeeping protein expression was done for TXNIP and $TXN_2$.

*2.6.3.3. Assessment of the pancreatic expression of miRNA 200b, BAX, and Bcl2 by quantitative real-time-PCR.* Tissue homogenate was processed for RNA extraction followed by Reverse Transcriptase (for cDNA synthesis) and quantitative real-time PCR (GeneCopoeia, Rockville, USA). Total RNA was extracted from homogenized tissues of all different groups with Direct-zol RNA Miniprep Plus(Cat# R2072, ZYMO RESEARCH CORP. USA and then quantity and quality were assessed by Beckman dual spectrophotometer. Sure script IV One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) was utilized for reverse transcription of the extracted RNA followed by PCR. The denaturation of samples was done as follows: 10 min at 45 °C for reverse transcription, 2 min at 98 °C for RT inactivation, and initial denaturation by 40 cycles of 10 s at 98 °C, 10 s at 55 °C and 30 s at72 °C for the amplification step. Regarding pancreatic BAX and BCl2, the extracted RNA (1 μg) was used for cDNA conversion using a high-capacity cDNA reverse transcription kit (#K4374966, Thermo Fisher Scientific, USA). Using an Applied Biosystem and software version 3.1 (StepOneTM, USA), real-time qPCR amplifier and descriptive analysis were carried out *via a* Two-step cycling protocol composed of initial denaturation at 95 °C for10 min, then denaturation at 95 °C for 10s and the annealing temperature was 60 °C for 60s. After the RT-PCR run, the data were expressed in Cycle threshold (Ct). The fold change in the relative expression of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. The housekeeping U6 and GAPDH were used as internal controls for miRNA 200b and BAX and Bcl2 genes respectively. The primer sequence is listed in Table .1.

*2.7. Histopathological examination of the pancreatic tissue*

An investigator who was blinded to the identity of the samples conducted all pathological evaluations to remove any potential for bias.

*2.7.1. Hematoxylin and eosin evaluation*

To determine the histopathological alterations, pancreatic tissues were removed, cleaned in a phosphate buffer solution containing 0.16 mg/ml to remove any erythrocytes, dried, and then fixed in formalin-buffered saline for 72 h before being implanted into paraffin. Transverse sections that were 8 m thick were then stained with hematoxylin and eosin (H&E). For histological investigation, photomicrographs were created utilizing a full HD microscopic camera run by a Leica application module (Leica Microsystems GmbH, Wetzlar, Germany).

**Table (1)**
Primers Sequence for miRNA200b, BAX, and Bcl2 that were used for real-time PCR.

| Gene | Primer sequence 5′-3′ |
|---|---|
| **miRNA 200b** | F: **CTC CCT AAA GCC TCC CACC** |
| | R: uni-miR qPCR primer included within the kit |
| **Housekeeping U6 gene** | F: **GTG CAG GGT CCG AGG T** |
| | R: **CGC TTC GGC AGC ACA TAT A** |
| **BAX** | **F: GGCCGGGTTGTCGCCCTTTT** |
| | **R: CCGCTCCCGGAGGAAGTCCA** |
| **Bcl2** | **F: CGGGAGATGTCGCCCCTGGT** |
| | **R: GCATGCTGGGGCCGTACAGT** |
| **Housekeeping GAPDH gene** | **F: 5′-TGGCATTGTGGAAGGGCTCA-3′** |
| | **R: 5′TGGATGCAGGGATGATGTTCT-3′** |

F: Forward R: Reverse primer.

### 2.7.2. Immunohistochemical expression of insulin

On another set of paraffin sections of pancreatic tissues from various study groups, immunohistochemical tests were conducted to find insulin expression. Here, slices were incubated with a monoclonal antibody against insulin at 37 °C for 60 min and was administered at a dilution of 0.5–1 l1μl. The main antibody was a mouse monoclonal insulin antibody (Catalog No. MA5-12037, Lab vision Corporation, Fremont, CA, USA). The slices were washed in PBS and then incubated with the secondary biotinylated antibody for 60 min at 37 °C (Dako, Carpentaria, CA, USA). 3,3-diaminobenzidine (DAB) was utilized as a chromogen and counterstained with hematoxylin solution following conjugation with streptavidin-biotin-peroxidase complex. With the use of Image J software from the National Institutes of Health, Bethesda, MD, the immunological reactivity of insulin was displayed as the optical density of the positively stained patches in high-power (X400) microscopic fields that were randomly selected.

### 2.7.3. Histomorphometric study analysis

The obtained data were examined using an image analyzer computer system at Ain Shams University in Cairo, Egypt (Leica Qwin 500, Leica, Cambridge, England). Pancreatic sections were immunostained with H&E and underwent the testing. According to Noor et al., the islets were counted and assessed for each study group using lower power magnification in 10 distinct, randomly chosen microscopic fields. The results were represented as N/10 mm2 of the pancreatic parenchyma. Additionally, the number of insulin immunostained β-cells per islet (numerical density of β-cells/islet), the mean area percent of insulin immunostaining, and The islet area were estimated at high magnification.

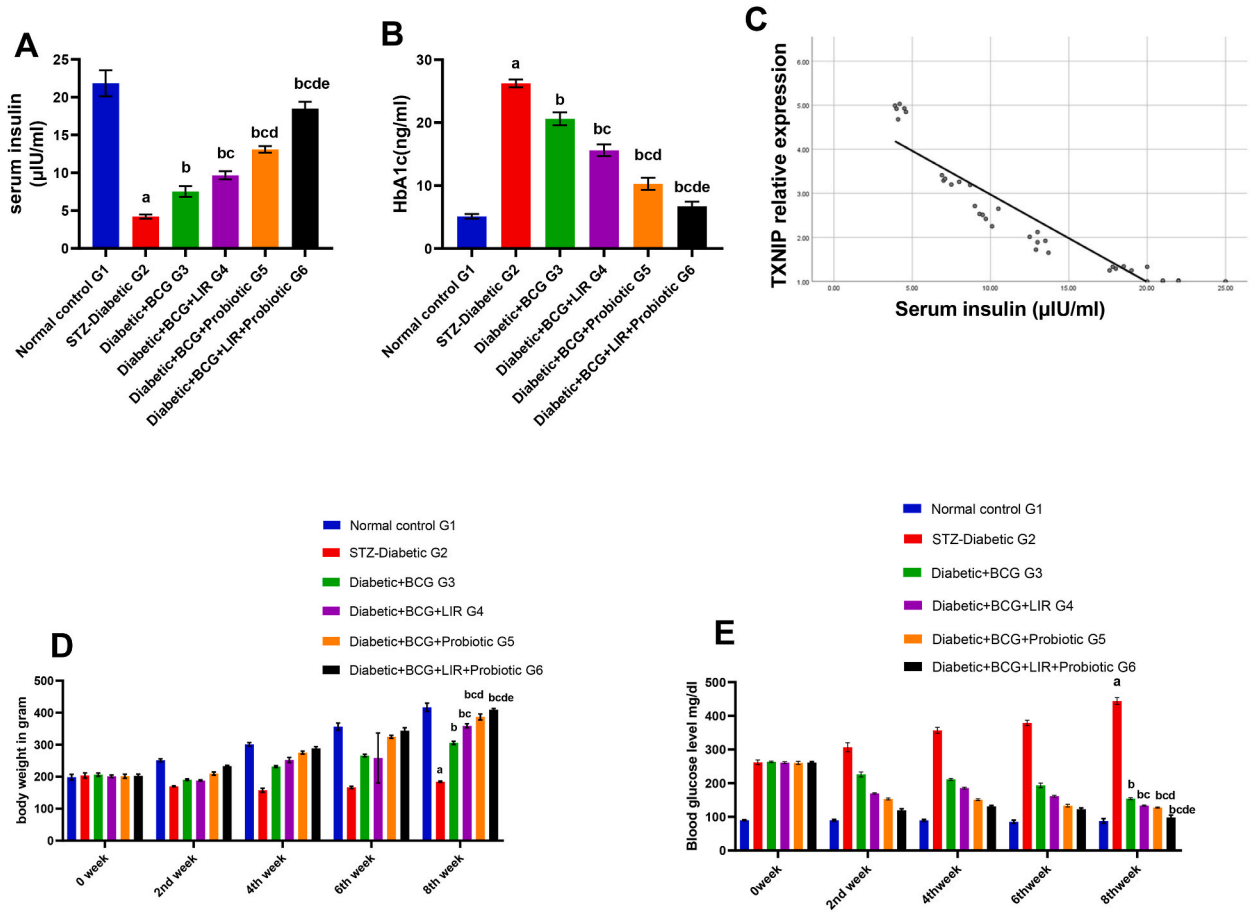



**Figure (1). The effect of BCG/Probiotic/Liraglutide on the body weight and glucose homeostasis parameters in the different studied groups for 8 weeks. (A): Serum insulin, (B): Glycosylated hemoglobin, (C): Correlation of serum insulin with the pancreatic TXNIP expression, (D): Body weight at the different time intervals, and (E): Fasting blood glucose at the different time intervals.** *Values represent means ± SD (n = 8) A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test apart from fasting blood glucose and bodyweight which was analyzed by two-way ANOVA followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

## 3. Statistical analysis

Data were analyzed using GraphPad prism Version 8. 4.3 Software (GraphPad Software; San Diego, CA, USA). The results are expressed as the mean (M) ± standard deviation for the normally distributed data. Normal distribution was assessed using the Shapiro–Wilk test. One-way Analysis of variance and post hoc tests were used to assess significant differences between the means of the measured parameters in the studied groups**.** Two independent variables, time, and the results of the treatment, had an impact on blood glucose and body weight. Accordingly, they were analyzed by 2-way ANOVA. The P-value of $<0.05$ was considered statistically significant. Correlations between quantitative variables were done using the Pearson correlation coefficient. P-values less than 0.05 were considered statistically significant.

## 4. Results

Notably, one- and two-way ANOVA revealed a significant difference between the diabetic non-treated group 2 and the normal control group 1 in all tested parameters at the different measuring intervals ($P < 0.0001$). Herein, all comparisons were between diabetic-treated groups with respect to their counterparts in the diabetic non-treated group, in addition to multigroup comparisons among diabetic-treated groups.

### *4.1. Effects of BCG/probiotic/liraglutide on mortality and body weight*

No mortality was recorded among rats of different groups. As shown in Fig. 1D and Table .2, at the 8th week, the diabetic-treated groups 3,4,5 and 6 demonstrated significant increases in body weight by about 65.7 %, 95.1 %, 100.9 %, and 122 % respectively compared to the diabetic non-treated group 2 ($P < 0.0001$). Notably, the combination of liraglutide or probiotic to BCG in groups 4 and 5 respectively, or the triple combination in group 6 showed a significant increase in the final body weight by about 17 %, 26 %, and 34 % respectively compared to BCG alone treated group 3 ($P < 0.0001$). Of note, as illustrated in Fig. 2A, there was a considerable surge in the mean relative difference of body weight in the different treated groups 3,4,5,6 by about 49.26 %,78.54 %,92.40 %, and 103.9 % respectively in contrast to their corresponding pretreatment weights at week 0. On the other hand, the diabetic untreated group exhibited an estimated decline in the mean relative difference of body weight by about 9.57 % in contrast to their corresponding pretreatment weights at week 0.

Interestingly, no significant difference was detected in the final body weight or the relative weight difference in the body weight between the triple combination group 6 and the normal control group ($P = 0.73$), ($P = 0.55$) respectively.

### *4.2. Effects of BCG/probiotic/liraglutide on glucose homeostasis parameters*

At the 8th week, the diabetic-treated groups 3, 4, 5, and 6 exhibited significant reduction ($P < 0.0001$) in FBG by about 65.13 %,70 %,71 %, and 77 % respectively when related to the diabetic non-treated group 2. Moreover, the combined BCG groups 4, 5, and 6 showed a remarkable decrease in FBG as compared to BCG alone treated group 3 ($P < 0.0001$) by about 13, 17 and 36 % respectively with even normalization of the values in the triple combination group 6. ($p = 0.18$) as shown in Fig. 1E and Table 3. Notably, as illustrated in Fig. 2B, there was a remarkable decline in the mean relative difference of blood glucose in the different treated groups 3,4,5,6 by about 41.54 %,48.89 %,51.88 %, and 62.53 % respectively in contrast to their corresponding pretreatment values at week 0. On the other hand, the diabetic untreated group exhibited an estimated up-leveling in the mean relative difference of blood glucose by about 69.54 % in contrast to their corresponding pretreatment weights at week 0.

Furthermore, there was a remarkable 0.7, 1.29, 2.10, and 3.37-fold increase in the serum insulin level in the diabetic-treated groups 3, 4, 5, and 6 respectively when compared to the diabetic untreated group 2 along with the parallel significant decrease in HBA1c by

**Table (2)**
The effect of BCG/Probiotic/Liraglutide on the body weight in the different studied groups.

| | Initial body weight at 0week (gm) | Final body weight at 8th week (gm) | Mean relative difference of body weight at the end of 8th week (%) |
|---|---|---|---|
| **Normal control group1** | 198.39 ± 8.71 | 416.94 ± 13.03 | 111 ± 14.79 |
| **STZ-Diabetic group 2** | 203.85 ± 8.18 | 184.38 ± 2.15 [a] | −9.570 ± 3.748 [a] |
| **Diabetic + BCG treated group 3** | 206.52 ± 5 | 305.8 ± 4.88 [ab] | 49.26 ± 3.122 [ab] |
| **Diabetic + BCG + LIR treated group 4** | 201.2 ± 4.03 | 359.12 ± 6.55[abc] | 78.54 ± 4.201 [abc] |
| **Diabetic + BCG + Probiotic treated group 5** | 201.5 ± 6.31 | 386.71 ± 9.34 [abcd] | 92.40 ± 5.446 [abcd] |
| **Diabetic + BCG + LIR + Probiotic treated group 6** | 202.55 ± 5.44 | 409.31 ± 3.72 [bcde] | 103.9 ± 5.924[bcd] |

*Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by two -way ANOVA followed by post hoc Tukey's test. The significance for Mean relative difference of body weight was determined using one-way ANOVA followed by post hoc Tukey's test. a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group. STZ: Streptozocin, LIR: liraglutide.

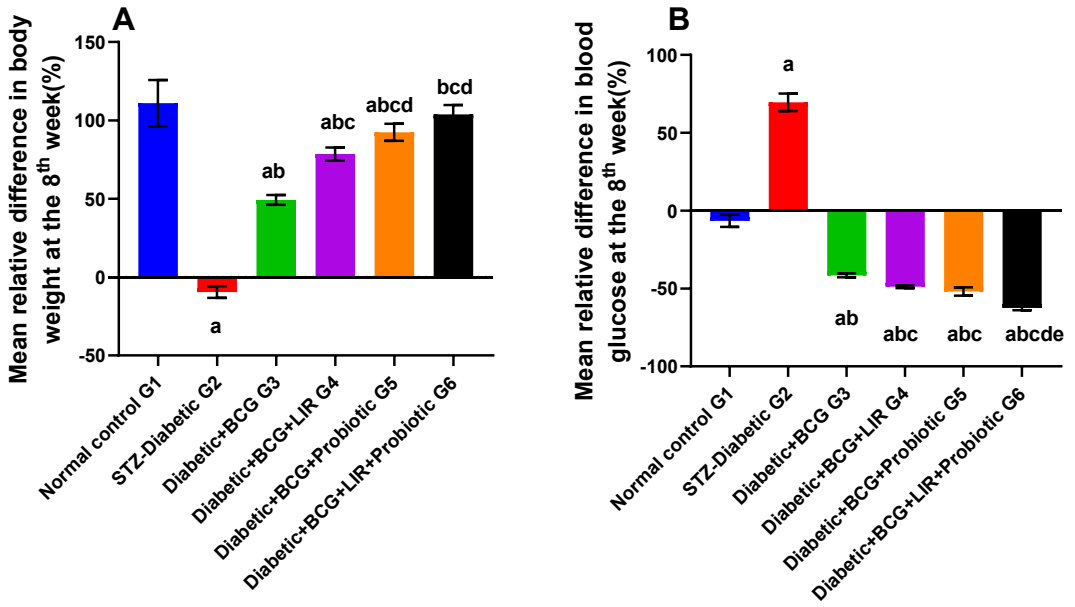



**Figure (2). The effect of BCG/Probiotic/Liraglutide on the mean relative difference in the body weight and blood glucose in the different studied groups at the 8th week. (A): relative difference in body weight, (B): relative difference in blood glucose.** *Values represent means ± SD (n = 8) A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

**Table (3)**
The effect of BCG/Probiotic/Liraglutide on glucose homeostasis markers in the different studied groups.

| | Fasting Blood glucose (mg/dl) at week 0 | Fasting Blood glucose (mg/dl) at 8th week | Mean relative difference of blood glucose at the end of 8th week (%) | Serum insulin (μIU/ml) | Glycosylated hemoglobin (HbA1c) |
|---|---|---|---|---|---|
| **Normal control group1** | 90 ± 1.41 | 84.65 ± 5.07 | −6.500 ± 3.937 | 21.83 ± 1.72 | 5.11 ± 0.36 |
| **STZ-Diabetic group2** | 262 ± 6.84 [a] | 444 ± 10.58 [a] | 69.54 ± 5.661 [a] | 4.22 ± 0.28 [a] | 26.23 ± 0.62 [a] |
| **Diabetic + BCG treated group3** | 263.5 ± 2.26 [a] | 154 ± 2.97 [ab] | −41.54 ± 1.213 [ab] | 7.53 ± 0.7 [ab] | 20.62 ± 1.02 [ab] |
| **Diabetic + BCG + LIR treated group4** | 261 ± 2.83 [a] | 133.33 ± 1.97 [abc] | −48.89 ± 0.9054 [abc] | 9.68 ± 0.55 [abc] | 15.62 ± 0.93 [abc] |
| **Diabetic + BCG + Probiotic treated group5** | 260.33 ± 4.41 [a] | 127.83 ± 1.6 [abcd] | −51.88 ± 2.638 [abc] | 13.1 ± 0.43 [abcd] | 10.28 ± 0.94 [abcd] |
| **Diabetic + BCG + LIR + Probiotic treated group6** | 261.5 ± 2.43 [a] | 98.33 ± 6.86 [bcde] | −62.53 ± 1.362 [abcde] | 18.48 ± 0.9 [abcde] | 6.700 ± 0.74 [abcde] |

*Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA apart from FBG was determined by two-way ANOVA test followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

about 21 %, 40 %, 60 % and 74 % respectively ($P < 0.0001$) as shown in Fig. 1A and Table 3. Noteworthy, the BCG combined groups 4, 5, and 6 elicited a significant increase in serum insulin level by about 28.5 %, 73.9 %, and 145 % respectively with a subsequent significant reduction in HbA1c by about 24.2 %,50 %, and 67.5 % respectively when compared to the BCG alone treated group 3 ($P < 0.0001$). Fig. 1B and Table 3.

### *4.3. Effects of BCG/probiotic/liraglutide on pancreatic oxidative stress biomarkers*

Perturbation of redox balance is a key player in beta cell dysfunction. The diabetic-treated rats succeeded in abating the oxidative stress denoted by a significant decrease in the pancreatic tissue level of MDA which was even normalized in group 6 ($P = 0.154$) in contrast to the normal control group(Fig. 3A). On the other hand, the pancreatic tissue levels of the antioxidant enzymes; GSH, SOD and CAT were significantly up-leveled in the diabetic-treated groups 3,4,5 and 6 as compared to their corresponding values in the

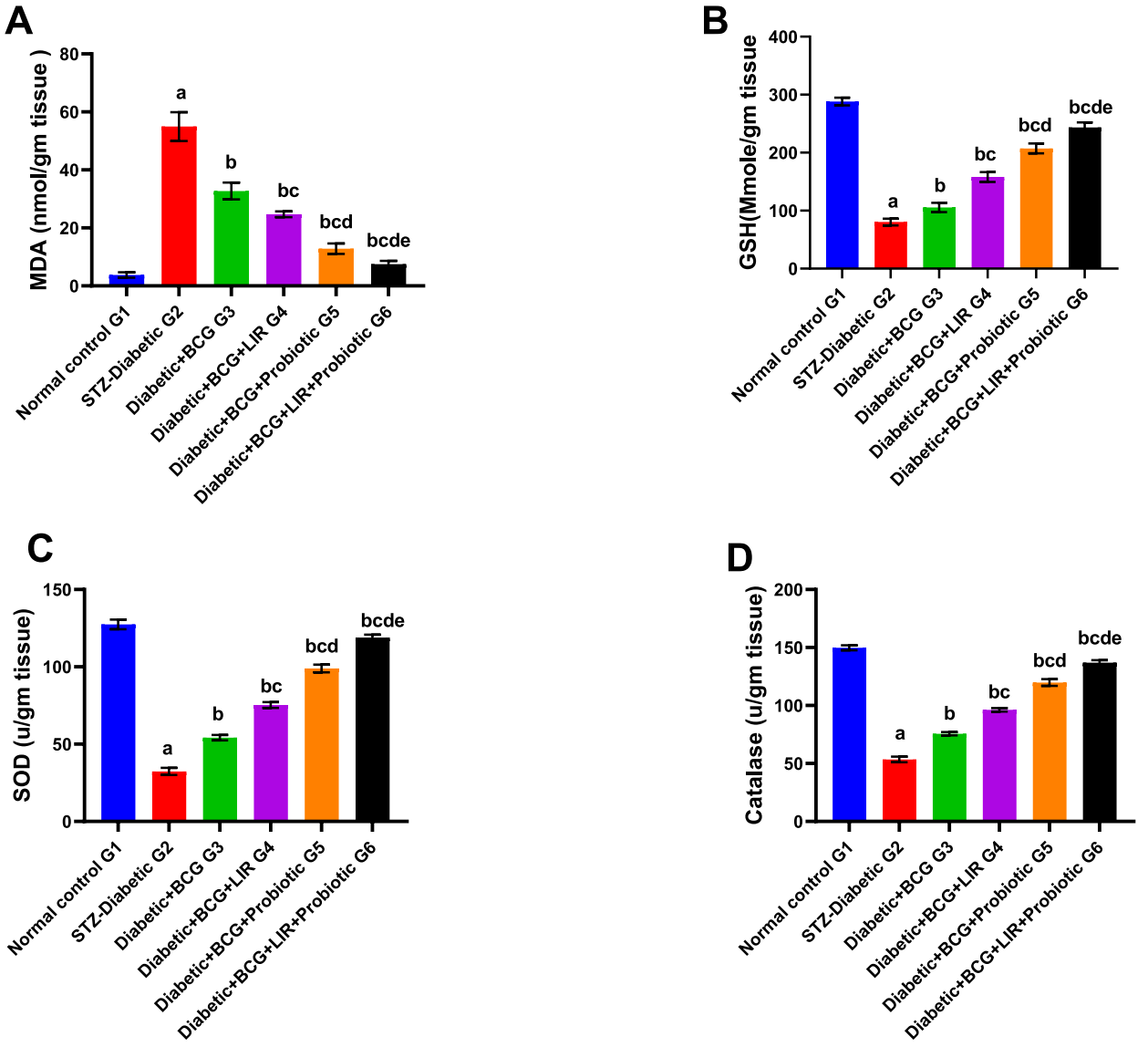



**Figure (3). The effect of BCG/Probiotic/Liraglutide on the pancreatic oxidative stress markers in the different studied groups for 8 weeks. (A): MDA, (B): GSH, (C): SOD, and (D): Catalase.** *Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

diabetic untreated group 2 ($P < 0.0001$). Noteworthy, the most obvious improvement in the redox mechanism was attained by the triple combined therapy in group 6 as depicted in Table 4 and Fig. 3B, C and 3D.

### 4.4. *Effects of BCG/probiotic/liraglutide on pancreatic inflammatory biomarkers*

Upregulation of NF-κB and TNF-α in oxidatively stressed pancreatic tissue is associated with NLRP3 inflammasome activation and subsequent release of active IL- β with substantial triggering of inflammatory milieu that ends with beta cell loss [28]. Results of the present study revealed a significant increment in pancreatic levels of NF-κB, TNFα, NLRP3, and IL- β in the diabetic untreated group 2. Conversely, all the diabetic-treated groups demonstrated a remarkable decrement ($P < 0.0001$) in these inflammatory markers.

Interestingly, significant suppression of the inflammatory milieu was observed in the combined BCG groups 4, 5, and 6 compared to BCG alone treated group 3 ($P < 0.0001$). The triple combined therapy in group 6 succeeded in achieving the lowest level of inflammatory markers and their levels were near to the corresponding level in the normal control group Table 5 and Fig. 4A,B,4C and 4D.

**Table (4)**
The effect of BCG/Probiotic/Liraglutide on the pancreatic oxidative stress markers in the different studied groups.

| | MDA (nmol/gm) | SOD activity (u/gm) | GSH (Mmole/gm) | CAT activity (u/gm) |
|---|---|---|---|---|
| **Normal control group1** | 3.76 ± 0.94 | 127.43 ± 3.15 | 288.26 ± 6.67 | 149.78 ± 2.12 |
| **STZ-Diabetic group 2** | 54.95 ± 4.98 [a] | 32.33 ± 2.31 [a] | 80.35 ± 5.77 [a] | 53.46 ± 2.45 [a] |
| **Diabetic + BCG treated group 3** | 32.72 ± 2.9 [ab] | 54.19 ± 1.74 [ab] | 105.49 ± 7.9 [ab] | 75.53 ± 1.31 [ab] |
| **Diabetic + BCG + LIR treated Group 4** | 24.68 ± 1.0 [abc] | 75.32 ± 1.92[abc] | 158.01 ± 8.6 [abc] | 96.29 ± 1.5 [abc] |
| **Diabetic + BCG + Probiotic treated. Group 5** | 12.85 ± 1.79 [abcd] | 98.93 ± 2.52 [abcd] | 207.2 ± 8.49 [abcd] | 119.83 ± 2.87 [abcd] |
| **Diabetic + BCG + LIR + Probiotic treated group 6** | 7.48 ± 1.16 [bcde] | 118.94 ± 1.82 [abcde] | 243.35 ± 8.65 [abcde] | 136.99 ± 2.15 [bcde] |

*Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test. a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

**Table (5)**
The effect of BCG/Probiotic/Liraglutide on the pancreatic inflammatory markers in the different studied groups.

| | TNFα (pg/mg tissue) | IL-1β (pg/mg tissue) | pS536-NFκB-p65 (pg/mg tissue) | NLRP3 (ng/mg tissue) |
|---|---|---|---|---|
| **Normal control group1** | 13.23 ± 1.43 | 23.28 ± 1.59 | 1.61 ± 0.04 | 0.43 ± 0.02 |
| **STZ-Diabetic group 2** | 86.7 ± 4.86 [a] | 83.58 ± 2.74 [a] | 8.38 ± 0.43 [a] | 2.9 ± 0.05 [a] |
| **Diabetic + BCG treated group 3** | 59.85 ± 2.32 [ab] | 58.38 ± 2.17 [ab] | 5.95 ± 0.25 [ab] | 1.92 ± 0.02 [ab] |
| **Diabetic + BCG + LIR treated group 4** | 41.5 ± 2.29 [abc] | 45.77 ± 1.59 [abc] | 4.39 ± 0.12 [abc] | 1.58 ± 0.04 [abc] |
| **Diabetic + BCG + Probiotic treated group 5** | 29.4 ± 1.81 [abcd] | 37.24 ± 1.55 [abcd] | 3.72 ± 0.11 [abcd] | 1.35 ± 0.03 [abcd] |
| **Diabetic + BCG + LIR + Probiotic treated group 6** | 19.17 ± 3.31 [abcde] | 28.33 ± 2.06 [abcde] | 2.85 ± 0.05 [abcde] | 1.2 ± 0.18 [abcde] |

*Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test. a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

### *4.5. Effects of BCG/probiotic/liraglutide on pro-apoptotic markers*

There is an intriguing link between TXNIP expression and beta cell inflammation and apoptosis [29]. Herein, all the diabetic-treated groups showed a significant downregulation of pancreatic TXNIP, phosphorylated ASK-1, and caspase-3 expression along with the parallel significant increase in TXN2 expression compared to their corresponding values in the diabetic non-treated group 2 ($P < 0.0001$). Notably, the combination of BCG with either probiotic and or liraglutide was superior to BCG alone treatment in group 3($P < 0.0001$) especially the triple therapy in group 6, succeeded in near normalization of the pancreatic TXNIP and phosphorylated ASK-1, upregulation of TXN2 and effectively restored the pancreatic caspase-3 to its level in the normal control group ($P = 0.24$) as demonstrated in Table 6 and Fig. 5A and B,5 D,5F and 5G(**supplementary file2**). More interestingly, pancreatic TXNIP expression was negatively correlated to serum insulin ($r = -0.927$ and $P < 0.001$) signifying its devastating role in beta cell dysfunction as shown in Fig. 1C.

Additionally, the diabetic-treated groups 3, 4, 5, and 6 exhibited significant amelioration in the pancreatic expression of IAPP and miRNA200b ($P < 0.0001$) compared to the diabetic non-treated group 2. Noteworthy, the combined BCG-treated groups 4, 5, and 6 showed a remarkable decrease in IAPP and miRNA200 expression in contrast to the BCG alone in group 3 ($P < 0.0001$) and their expression in group 6 approximately reached their corresponding values in the normal control group ($P = 0.05$) and ($P = 0.06$) for miRNA and IAPP respectively as shown in Table 6 and Fig. 5 Eand 5F 5H.

Moreover, the proapoptotic effect of TXNIP overexpression and its related aforementioned apoptotic markers was supported by the significant 28-fold rise in BAX/Bcl2 ratio in the diabetic untreated group 2 when compared to the normal control group ($P < 0.0001$) On the other hand, all diabetic-treated groups 3,4,5 and 6 demonstrated a significant reduction in BAX/Bcl2 ratio by about 0.59 %,83.5 %,91 %, and 95.32 % respectively as compared to their counterparts in the diabetic non-treated group 2 ($P < 0.0001$). Notably, combining BCG with liraglutide and probiotic in group 6 succeeded in bringing the BAX/Bcl2 ratio back to its normal balance as compared to the control group ($P = 0.957$). Fig. 5I.

### *4.6. Effects of BCG/probiotic/liraglutide on histological examination of islet cells and pancreatic acini by hematoxylin and eosin*

H & E-stained pancreatic sections of the control group showed abundant normal pancreatic islets and cellular architecture in the form of pale rounded intact islets of Langerhans surrounded by acinar cells. On the other hand, in the diabetic non-treated group 2, the islets were comparatively atrophic, and shrunken and showed prominently vacuolar degeneration, hypereosinophilic cytoplasm, areas of hemorrhage, and severely congested blood vessels in between. Conversely, BCG-treated group 3 showed partially recovered islets from the previously noted vacuolar degeneration with less intense vascular congestion. Moreover, combining liraglutide and probiotic to BCG in groups 4 and 5 respectively, restored the islet size and their normal microstructural organization in addition to prominent ductal proliferation with fewer areas of vacuolar degeneration. Surprisingly, the triple combination therapy in group 6 showed almost

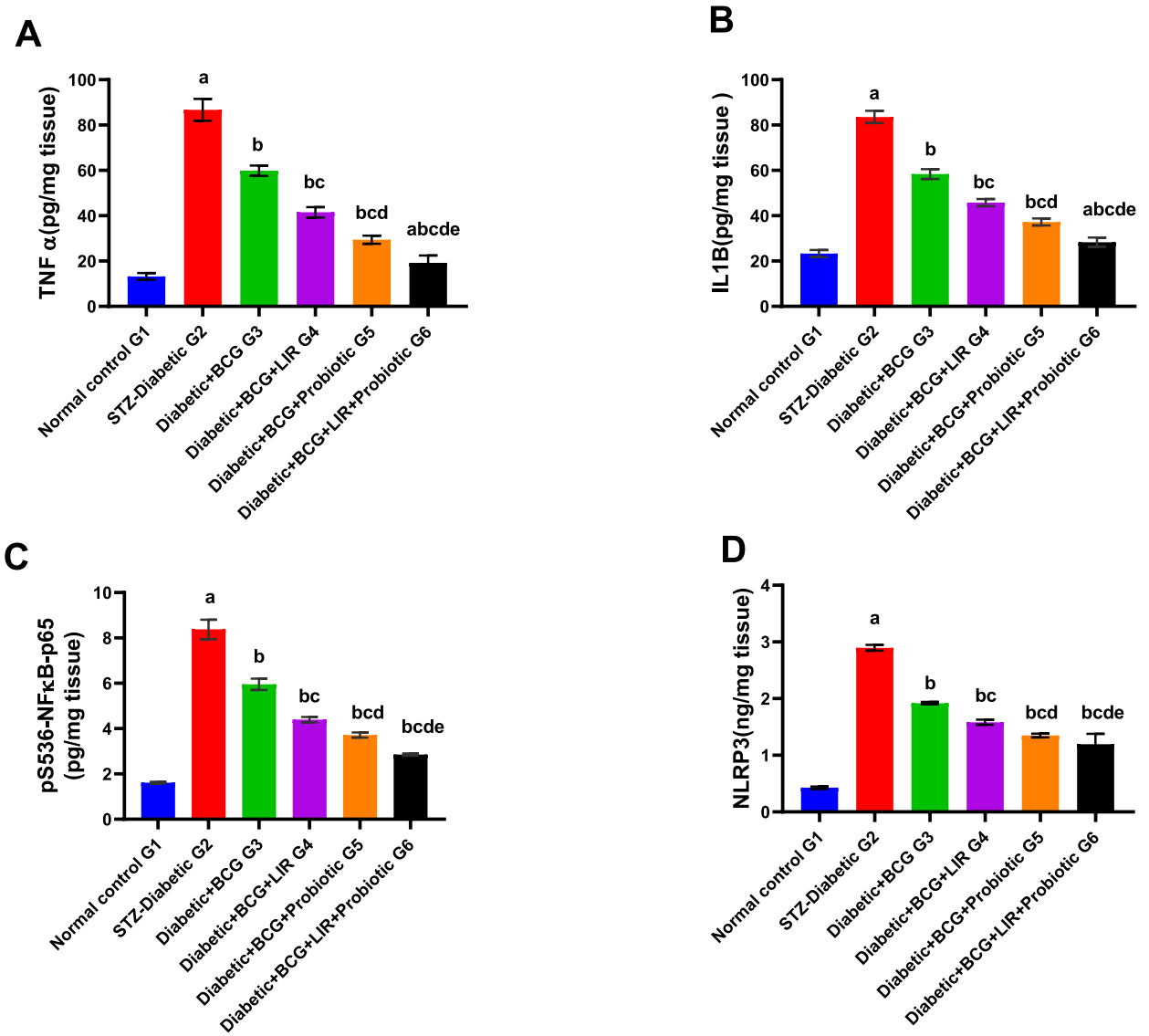



**Figure (4). The effect of BCG/Probiotic/Liraglutide on the pancreatic inflammatory markers in the different studied groups for 8 weeks. (A): TNFα, (B): IL-1β, (C): pS536-NFκB-p65 *(D):* NLRP 3.** *Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group. STZ: Streptozocin, LIR: liraglutide.

normal microarchitecture and cytology with almost closely packed islets and normal islets size. There were no areas of vacuolar degeneration, vascular congestion, or areas of inflammatory infiltration as displayed in Fig. 6a-f(supplementary file3**).**

### *4.7. Effects of BCG/probiotic/liraglutide on the number of cells per islet, islet cell area, and number of islets*

Histomorphometric evaluation of the number of cells per islet, islet cell area, and the number of islets revealed a significant increase in these parameters in all diabetic-treated groups ($P < 0.0001$) compared to the diabetic non-treated group 2. Notably, combining liraglutide and/or probiotic with BCG showed a highly significant increase in all these morphometric parameters when compared to BCG monotherapy in group 3. Interestingly, the triple combination therapy in group 6 exhibited near-normal values of these parameters which was evidenced by the remarkable increase in the number of islets that was nearly comparable to its corresponding value in the normal control group ($p = 0.99$). Table 7 and Fig. 7A and B and 7C.

**Table (6)**
The effect of BCG/Probiotic/Liraglutide on the pancreatic apoptotic markers in the different studied groups.

| | miRNA200b/U6 (AU) | TXN2/GAPDH (AU) | TXNIP/ GAPDH (AU) | IAPP/βActin (AU) | p-ASKThr838/ βActin (AU) | Caspase 3 activity (ng/mg tissue) |
|---|---|---|---|---|---|---|
| **Normal control Group 1** | 1.01 ± 0.012 | 1.01 ± 0.01 | 1.01 ± 0.01 | 1.01 ± 0.01 | 1.01 ± 0.01 | 0.58 ± 0.06 |
| **STZ-Diabetic group 2** | 3.74 ± 0.138 [a] | 0.21 ± 0.03 [a] | 4.9 ± 0.12 [a] | 5.93 ± 0.44 [a] | 4.86 ± 0.15 [a] | 6.63 ± 0.29 [a] |
| **Diabetic + BCG treated group 3** | 2.70 ± 0.147 [ab] | 0.46 ± 0.04 [ab] | 3.28 ± 0.08 [ab] | 3.77 ± 0.24 [ab] | 3.39 ± 0.03 [ab] | 4.18 ± 0.12 [ab] |
| **Diabetic + BCG + LIR treated group 4.** | 1.92 ± 0.071 [abc] | 0.63 ± 0.03 [abc] | 2.51 ± 0.16 [abc] | 2.6 ± 0.33 [abc] | 2.62 ± 0.1 [abc] | 3.08 ± 0.15 [abc] |
| **Diabetic + BCG + Probiotic treated group 5** | 1.58 ± 0.073 [abcd] | 0.75 ± 0.04 [abcd] | 1.89 ± 0.18 [abcd] | 1.88 ± 0.15 [abcd] | 1.75 ± 0.08 [abcd] | 2.1 ± 0.23 [abcd] |
| **Diabetic + BCG + LIR + Probiotic treated group 6** | 1.77 ± 0.051 [bcde] | 0.93 ± 0.04 [abcde] | 1.3 ± 0.04 [abcde] | 1.45 ± 0.08 [bcde] | 1.24 ± 0.04 [abcde] | 0.8 ± 0.06 [bcde] |

*Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test. a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/ LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

### *4.8. Effects of BCG/probiotic/liraglutide on the percentage of insulin immunoreactivity*

Immunostained pancreatic sections of beta cells as shown in Table 7 and Fig. 8, shows a significant increase in the % of insulin immunoreactivity in the diabetic-treated groups 3, 4, 5, and 6 by about 28.82 %, 46.56 %, 46.79 %, and 61.82 respectively ($P < 0.0001$) compared to diabetic non-treated group 2. Additionally, combining liraglutide and/or probiotic to BCG in groups 4, 5, and 6 was more effective in increasing the density of immunoreactivity compared to the BCG monotherapy group ($P < 0.0001$) by about 14.06 %, 14.21 %, and 25.58 % respectively. Noteworthy, beta cell immunostaining in the triple combination therapy was nearly comparable to that in the normal control group ($p = 0.17$). Fig. 7D.

## 5. Discussion

Diabetes remains a public health challenge, especially among young populations. The diabetes frequency has significantly elevated in both developing as well as developed countries over the preceding few decades, resulting in a serious economic burden [30] and becoming an important global health priority [31].

Despite being adversely associated with a number of side effects, such as dyslipidemia, hypoglycemia, insulin resistance, and weight gain, insulin therapy is still the primary treatment for type 1 diabetes (T1DM) [32]. Although insulin analog formulation and delivery have advanced recently, most T1DM patients still do not have appropriate glycemic control [33]. Non-insulin-dependent glucose-lowering approaches could offer a way to improve glycemic control in individuals without hypoglycemia or weight gain [34].

Recently, it has been discovered that T1DM patients still possess functioning β cells. These remaining beta cell mass at the onset is highly influenced by the degree of pancreatic beta cell inflammation [35]. Previous studies suggested that the progressive loss of beta cells after the onset of overt type 1 diabetes is partially attributed to the ongoing autoimmune inflammatory drive and being overwhelmed by the hyperglycemic state that resulted in ER stress and apoptosis [36]. Therefore, there is an urgent need for innovative strategies that could protect against β-cell death induced by apoptosis and increase pancreatic β-cell survival at the onset of the disease [37], especially in the evolving era of regenerative medicine.

The current study was planned to evaluate the potential synergistic impact of combining either liraglutide and/or probiotic with BCG vaccine on the pancreatic function and structure inT1DM rat model. Although the desirable effect of each of the BCG vaccine, liraglutide, and probiotics in T1DM has been noted in the literature, their concomitant administration & comparison of their effects has not been yet evaluated, to the appropriate of the authors' knowledge, their possible interaction with pyroptosis and inflammasome in TXNIP/NLRP3/IL1β signaling pathway has not yet been investigated.

Streptozotocin (STZ) has been used in the current study to establish a rat model of T1DM. Exposure to low doses of STZ was reported to cause pancreatitis and complex immune response against β cells resembling that seen in type 1 DM, resulting in sustained hyperglycemia, and a marked reduction in insulin biosynthesis and secretion from islet β-cells [38].

Unexpectedly, it was noted that humans might be intermittently in contact with STZ *via* opportunistic infections with the STZ-producing bacteria and/or *via* certain food products that include STZ. Moreover, the existence of STZ-producing bacteria in the gut microbiota of some individuals might be another source of long-term STZ exposure [39].

STZ-induced diabetic animal model is a valuable platform for understanding beta-cell glucotoxicity. It has been used in the present study to induce a state of chronic hyperglycemia. Overloading on glucose can trigger several metabolic or signaling pathways, producing more reactive oxygen species and resulting in β cell damage and oxidative stress [40].

In the present study, STZ destroyed β-cells of islets of Langerhans in pancreatic tissue, resulting in a remarkable surge in FBG and HbA1c with a considerable decline in serum insulin. The deleterious effect on beta cells of the pancreas in the STZ-diabetic untreated group was confirmed by histopathological findings in the form of atrophic shrunken islets with intracellular vacuolations, pyknotic nuclei congested capillaries, and weak insulin immunostaining. Our finding was in accordance with previous results that showed the

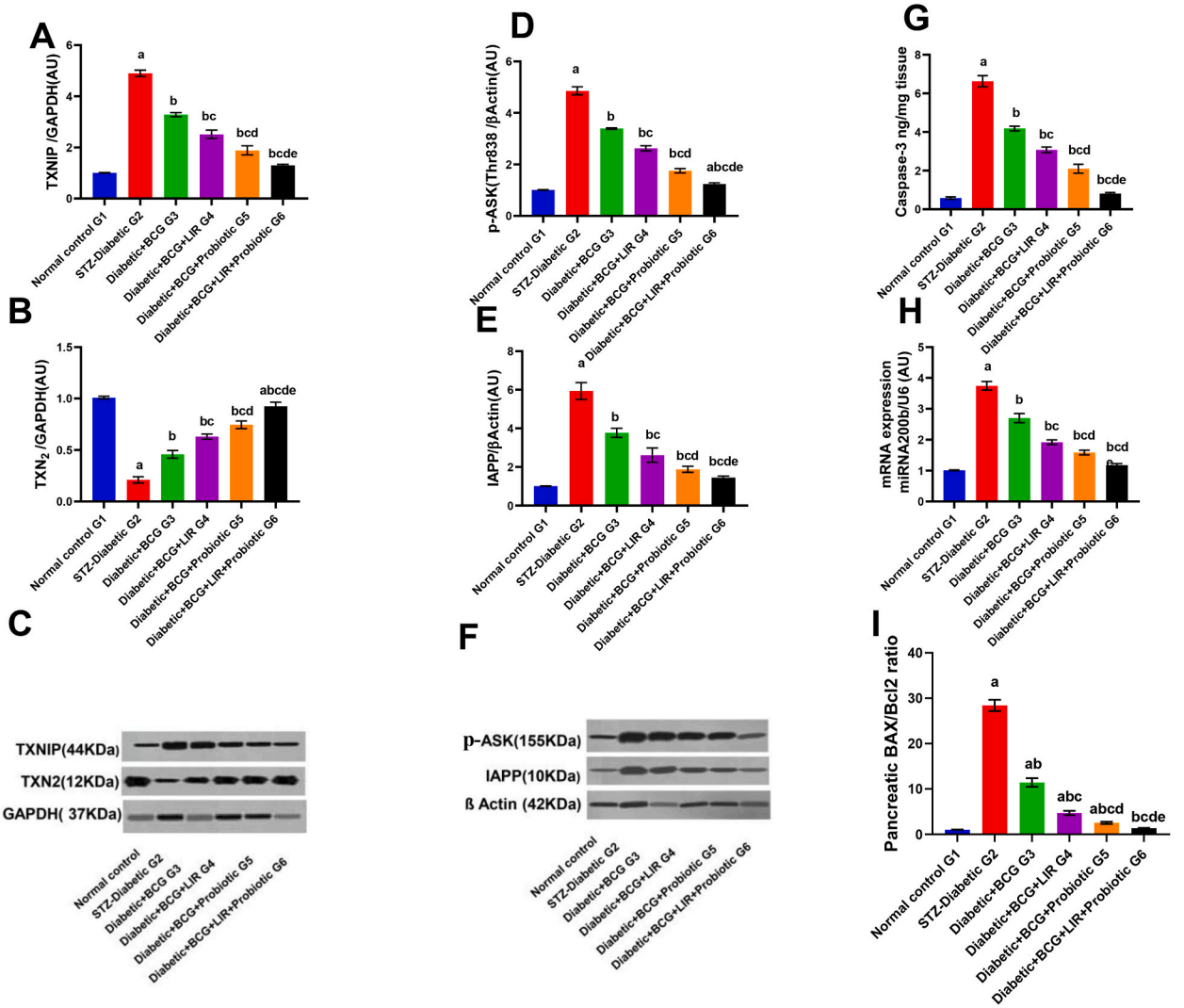



**Figure (5). The effect of BCG/Probiotic/Liraglutide on the pancreatic apoptotic markers in the different studied groups for 8 weeks. (A): Pancreatic expression of TXNIP, (B): Pancreatic expression of TXN2, (C): Densiometric expression of TXNIP and TXN2, (D): Pancreatic expression of the phosphorylated form of ASK, (E): Pancreatic expression of IAPP, (F): Densiometric expression of p-ASK and IAPP, (G): Pancreatic level of Caspase-3, (H): Pancreatic mRNA expression of miRNA200b, and (I): Pancreatic BAX/Bcl2 ratio.** *Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group. STZ: Streptozocin, LIR: liraglutide.

ability of STZ to induce similar microscopic changes in the pancreas in the form of islet atrophy with apoptotic cellular alterations and vacuolar changes in some sections of weak insulin immunoreactions [41]. The altered pancreatic histoarchitecture was reflected in a remarkable surge in FBG and HbA1c with a considerable decline in serum insulin.

In this study, treatment with BCG vaccine, liraglutide, and/or probiotic alone or in combination significantly reduced blood glucose level and HbA1c with a parallel increase in insulin level. Moreover, the triple combination therapy was the most effective in controlling hyperglycemia and building up β-cells. mass Moreover, the diabetic untreated group demonstrated a significant weight loss at the end of the experiment, compared to the control group. However, all treated diabetic groups showed a significant increase in body weight. Notably, the triple combination therapy was the most effective in maintaining body weight. Results of the present study could be explained by the marked improvement induced by the combination therapy in the pancreatic cytoarchitecture with even restoration of the islet histoarchitecture with a dense cellular population in the triple combination therapy group 6. This was asserted by the remarkable elevation in the serum insulin level along with a parallel significant decrease in HBA1c and FBG.

Undoubtedly, dysbiosis or imbalance of microbes in the gut has been associated with susceptibility and progression of T1DM [42]. Several studies have reported an altered gut microbiota in T1DM patients. De Goffau et al. [43] analyzed the gut microbiota at the

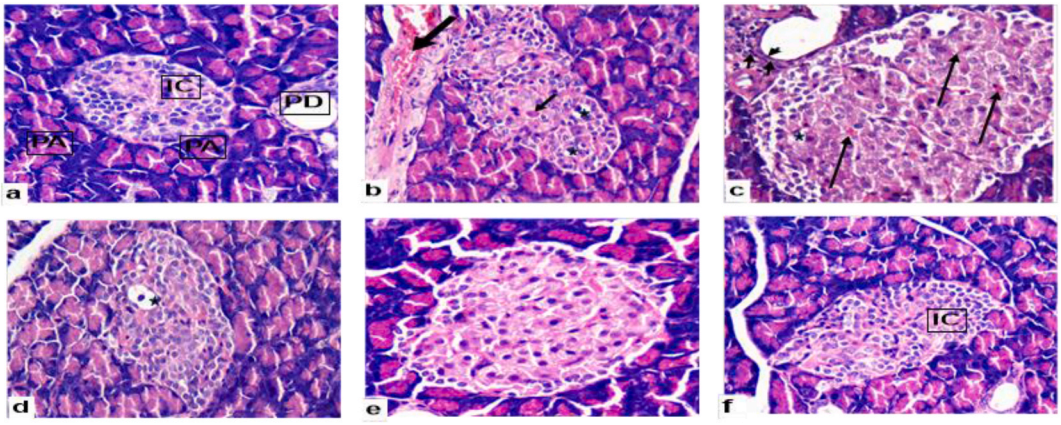



**Figure (6). photomicrographs of pancreatic tissues of the different studied groups. stained with H &E** (a): Control group shows the normal structural arrangement of pancreatic acini (PA) and pancreatic duct (PD) and rounded intact islet cells(IC) of Langerhans. (b): STZ-diabetic-untreated group shows distorted islet cells with vacuolated cytoplasm (stars) and small pyknotic nuclei and acidophilic cytoplasm(thin black arrow) denoting apoptotic changes with areas of hemorrhage and severely congested blood vessels in between(thick black arrow). (c) The BCG-treated group shows an apparent increase in islet size along with ductal proliferation (head Arrow) and ductal epithelial stratification (short black arrows) were noted with areas of vascular congestion (thin long arrows) in addition to a few islet cells with vacuolated cytoplasm (star). (d): The BCG + LIR-treated group shows an apparent improvement in the islet size with normal cellular architecture but, some islet cells are distorted with vacuolated cytoplasm noted (star). (e): BCG + probiotic-treated group demonstrates an almost well arrangement of the cellular architecture of the pancreatic islet cells (IC)with densely populated pancreatic islets. (f): BCG + LIR + probiotic-treated group shows the maximum recovery from all histoarchitectural disarray. Magnification: × 400 and Scale bar = 25 μm. STZ: Streptozocin, LIR: liraglutide.

**Table (7)**
The effect of BCG/Probiotic/Liraglutide on the pancreatic morphometric studies in the different studied groups.

| | Number of cells per islet | Islet cell area (mm2) | Number of islets (N/mm2) | Percentage of insulin immunoreactivity |
|---|---|---|---|---|
| **Normal control group1** | 140.33 ± 4.41 | 0.0171 ± 0.0003 | 11.5 ± 0.84 | 94.52 ± 1.98 |
| **STZ-Diabetic group 2** | 61.5 ± 3.08 $^{a}$ | 0.0055 ± 0.0004 $^{a}$ | 3.67 ± 0.52 $^{a}$ | 56.82 ± 2.71 $^{a}$ |
| **Diabetic + BCG treated Group 3** | 86.83 ± 2.14 $^{ab}$ | 0.0126 ± 0.0002 $^{ab}$ | 6 ± 0.63 $^{ab}$ | 73.05 ± 1.82 $^{ab}$ |
| **Diabetic + BCG + LIR treated group 4** | 101.17 ± 1.83 $^{abc}$ | 0.0142 ± 0.0001 $^{abc}$ | 8.33 ± 0.52 $^{abc}$ | 83.33 ± 1.36 $^{abc}$ |
| **Diabetic + BCG + Probiotic treated group 5** | 123 ± 1.79 $^{abcd}$ | 0.0155 ± 0.0001 $^{abcd}$ | 9.33 ± 0.52 $^{abc}$ | 83.41 ± 1.36 $^{abc}$ |
| **Diabetic + BCG + LIR + Probiotic treated group 6** | 133.67 ± 3.08 $^{abcde}$ | 0.0164 ± 0.0002 $^{abcde}$ | 11.67 ± 0.52 $^{bcde}$ | 91.95 ± 1.25 $^{bcde}$ |

*Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test. a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group.STZ: Streptozocin, LIR: liraglutide.

onset of T1DM in young children, there was an upgrade in the level of *Bacteroidetes* and *Streptococcus mitis*. On the other hand, non-diabetic controls demonstrated a higher prevalence of *Lactobacillus plantarum* and *Clostridium* clusters that are high producers of butyrate, which has excellent anti-inflammatory properties. Moreover, Siljander et al. [44], reported that the high level of Bacteroidetes is a key player in initiating the inflammatory drive in the gut of T1DM children with substantial translocation of the inflammatory cytokines that activate toll-like receptor 4 resulting in metabolic inflammation [45]. Our result revealed that probiotic supplementation afforded a significant improvement in glucose homeostasis parameters. Consistent with our study, Syrianti, and his colleagues demonstrated that administration of *L. acidophilus* at both doses of $1.5x10^{8}$ and $1.5x10^{9}$ CFU/mL/day improved the pancreatic histopathology of type-1 DM rats induced by two doses of STZ(50 mg/kg/day) and decreased FBG [24].

Furthermore, a recent study on the therapeutic effect of *Lactobacillus brevis* done by Abdelazez et al. reported the significant reduction of FBG and the remarkable improvement of pancreatic histomorphic parameters along with the elevation of the serum insulin in STZ-induced T1DM in mice [46]. Moreover, the latest published meta-analysis and systematic review comprised five randomized controlled trials ($n = 356$; mean age = 11.7 years old). The authors investigated the impact of probiotic supplementation on hemoglobin A1c (HbA1c), fasting blood glucose (FBG), C-peptide, and insulin requirements in T1DM. The pooled effect size revealed a significant decrease in FBG. They recommended probiotics as a complementary therapeutic strategy in T1DM [47].

Our results are consistent with those from Baik's study which revealed that the administration of BCG injection just prior to STZ-induction of diabetes in mice, significantly reduced blood glucose levels, ameliorated insulitis scores, and prevented body weight loss

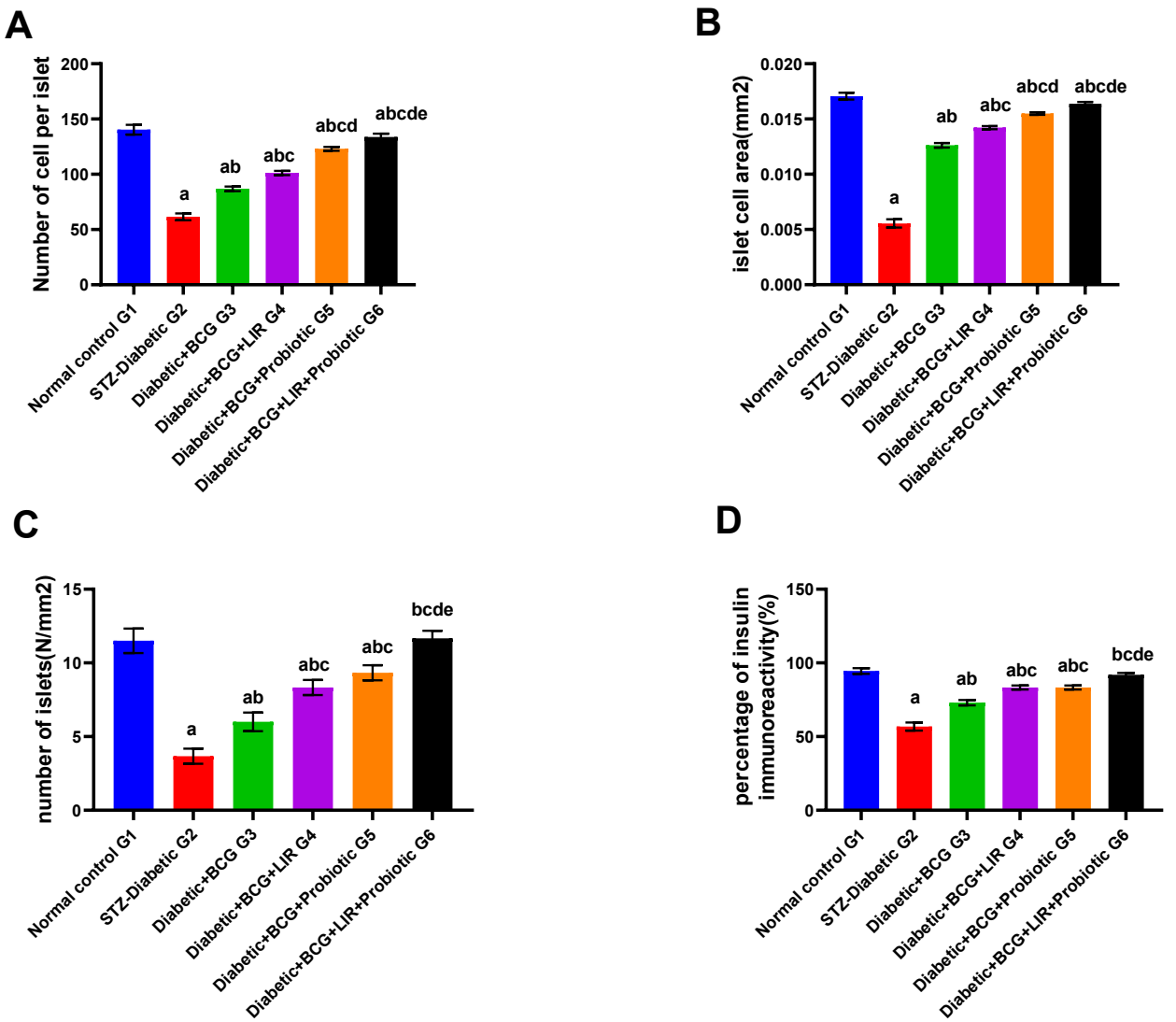



**Figure (7). The effect of BCG/Probiotic/Liraglutide on the pancreatic morphometric studies in the different studied groups for 8 weeks. (A): Number of cells per islet, (B): Islet cell area, (C): Number of islets, and (D): Percentage of insulin immunoreactivity.** *Values represent means ± SD (n = 8). A significant difference is reported when P is less than 0.05 and determined by one-way ANOVA followed by post hoc Tukey's test a*: statistically significant as compared to the normal control group. b: statistically significant as compared to the diabetic group. c: statistically significant as compared to the diabetic treated with BCG group. d: statistically significant as compared to the diabetic treated with BCG/LIR group. e: statistically significant as compared to the diabetic treated with BCG/Probiotic group. STZ: Streptozocin, LIR: liraglutide.

at the 61st of the study [48].

Notably, BCG treatment of T1DM patients was introduced relatively recently. Clinical benefits of the BCG vaccine have been demonstrated in T1DM, as one study linked lower incidence rates of T1DM to numerous childhood vaccinations [49]. In an 8-year prospective study, Kühtreiber and colleagues found that two intradermal doses of BCG given four weeks apart brought HbA1c levels in advanced T1DM patients close to normal after about three years of treatment. The beneficial effects continued for an additional five years, supporting the theory that the reduction in blood sugar levels was caused by both immune and metabolic effects, immunological system reset in the form of increased mRNA expression of suppressive T-regulatory (Treg) cells, and decreased autoreactive cytotoxic lymphocytes (CTLs) that target pancreatic β islet cells [50]. Even more authors speculated that even in progressed T1DM when the pancreas no longer has functional β islets, BCG treatment drops blood sugars *via* switches cellular glucose metabolism from primarily oxidative phosphorylation with low glucose utilization state to augmented early aerobic glycolysis state and shifting of glucose to the Pentose Phosphate Shunt for purine biosynthesis. These aforementioned clinical and experimental studies support our glucose homeostasis findings in BCG-treated diabetic groups.

Results of the present study are also in line with the previous study of Doyle and Egan who reported that chronic GLP-1 receptor agonist therapy has also been demonstrated to boost pancreatic beta cell mass in diabetic animal models [51]. A balance of cell growth,

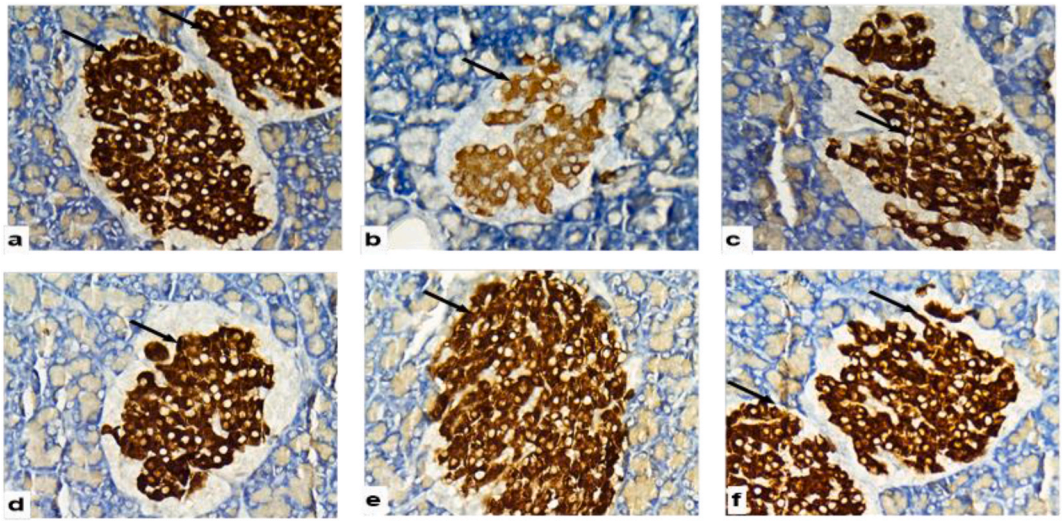



**Figure (8). Photomicrographs of the insulin immunostaining of pancreatic tissues from different studied groups.** Black arrows are indicative of positive insulin immunoreactivity. (a) Normal control group displays a strong insulin immunoreactivity of pancreatic islets. (b): STZ-diabetic-untreated group depicts shrunken islets with a marked decline in insulin immunoreactivity. (c) BCG-treated group, (d): BCG + LIR-treated group, (e): BCG + probiotic treated group, and (f): BCG + LIR + probiotic-treated - group demonstrates variable degree of enhanced insulin immunoreactivity which was prominently marked in the BCG + LIR + probiotic-treated group. STZ: Streptozocin, LIR: liraglutide.

cell death, and neogenesis is assumed to define beta-cell mass. Intriguingly, A recently published study by Villalba et al. [52], in STZ-induced diabetes in mice, reported that liraglutide significantly upregulates insulin gene, and succeeded in achieving normoglycemia during 30 days of continuous treatment with a considerable increase in β-cell mass detected by immunofluorescence. Both parameters were ameliorated upon its withdrawal. This was in accordance with our data regarding the significant improvement in pancreatic histomorphic parameters and insulin immunostaining in BCG + LIR and BCG + LIR + probiotic groups.,

Oxidative stress, resulted from decreased ROS scavenging capacity of the pancreas, and inflammation act as cooperative and synergistic partners in the pathophysiology of diabetes [53,54]. Several investigations have found that hyperglycemia contributes to the buildup of ROS and dysfunction of antioxidants in diabetic experimental animals and patients, resulting in a considerable increment in free radical generation, that ends eventually with overwhelming oxidative stress, especially with low expression of antioxidants in pancreatic tissues [6,55–57]. In line with these outcomes, our data revealed the presence of oxidative insult to β cells in the untreated diabetic group evidenced by the significant elevation in the pancreatic tissue level of MDA together with a significant reduction of the antioxidant enzymes; SOD, GSH, and catalase. However, all treated diabetic groups have restored most of the redox homeostasis in the pancreatic β cells but, the most obvious improvement was attained by the triple combined therapy in group 6.

In line with our results, the antioxidative properties of probiotics have been demonstrated in several studies [58,59]. The metabolites secreted by probiotics may play key roles in promoting antioxidative activity and protecting cells from oxidative damage. Also, liraglutide has been shown to exert a strong antioxidative effect in different tissues and maintain the balance of energy metabolism [60].

Additionally, El-shafey and his colleagues recently illustrated the significant effect of liraglutide on the restoration of redox balance in STZ-induced diabetes type-1 in rats [61]. Moreover, Elseady and his coworkers demonstrated that liraglutide succeeded in alleviating dexamethasone-induced pancreatic cytotoxicity by enhancing pancreatic anti-oxidative capacity *via* upregulation of nuclear factor erythroid 2–related factor 2 (Nrf2) [62]. The deleterious effect on β cells induced by oxidative stress in the STZ-diabetic group in this study resulted in a significant rise in the inflammatory markers TNFα, IL-1β, NLRP3, and NF-κB.

Our results are in accordance with that of Li X. et al. who suggested that activation of (NF-κB)-inducing kinase [9] is involved in B cell dysfunction [63]. In a similar manner, pro-inflammatory cytokines activate genes that draw macrophages to β cells, enclosing them in an inflammatory milieu [64]. Notably, the NLRP3 inflammasome stimulates the generation and release of proinflammatory cytokines, which can intensify inflammatory reactions. Numerous earlier investigations have demonstrated that NLRP3 is crucial to the development of T1DM. and this disease will progress more slowly if the NLRP3 gene is removed [65].

Previous studies have found that liraglutide can ameliorate inflammation and enhance the redox mechanism by inhibiting the levels of NF-κB which has a priming effect on NLRP3 inflammasome and enhances transcription of their components [66]. Suppression of β-cell apoptosis and increasing β-cell replacement could be aiding factors and support the role of liraglutide in β-cell mass restoration in type 1 diabetes [67].

It has been discovered that consuming probiotic strains increases the levels of anti-inflammatory cytokines like IL-10 and decreases the levels of pro-inflammatory cytokines like TNF, IL-1β, and IL-6. Probiotics have also been shown to increase GLP-1 production, which in turn stimulates pancreatic cell secretion of insulin and lowers blood sugar. Its capacity to elevate short-chain fatty acids provided an explanation for the incretin action. These data show how probiotics can help manage T1DM by reestablishing the gut

microbiota-immune axis' equilibrium [68]. Additionally, in line with our work, A recently published study reported that oral administration of *Lactobacillus Plantarum* treatment for 3 weeks in the STZ diabetic model in mice downregulated the pancreatic expression of NLRP3 and IL-β [69].

Our results revealed that the glycemic control of the group treated with BCG/probiotics was superior to BCG/liraglutide, this could be explained by the fact that probiotics besides their beneficial effect in regulating gut membrane integrity and permeability, probiotics were proved to have "incretin effect" which means its ability to elevation GLP-1 that stimulate insulin release, In addition, probiotics was found to elevate levels of anti-inflammatory cytokines as IL-10 and TGF- β and to reduce levels of pro inflammatory cytokines as IL-6 and TNF alpha and IL-1B [70].

Accordingly, the improvement of pancreatic histological findings as well as immunohistochemical results in the current study may be explained by the synergistic antioxidant and anti-inflammatory capacity provided by combining either liraglutide and/or probiotics with the BCG vaccine.

To explore and delve into the underlying mechanistic pathway that might be behind our results, we investigated the TXNIP/NLRP3 signaling pathway. The preservation of redox balance within the cell is largely dependent on the thioredoxin system (20). Two isoforms of thioredoxin have been found in human cells: thioredoxin 1 (TXN1) and thioredoxin 2 (TXN2) (11). Thioredoxin's oxidoreductase reduces oxidized proteins that are caused by ROS. As a component of the mitochondrial antioxidant defense system, TXNIP is translocated to the mitochondria where it interacts with mitochondrial TXN2. Apoptosis signal-regulating kinase 1 (ASK-1) is inhibited in the mitochondria by TXNIP, which does this by competing with ASK-1 to bind with TXN-2. This releases ASK-1 from TXN2's inhibition, allowing ASK-1 to be phosphorylated and activated, which in turn triggers the apoptotic signaling cascade in pancreatic β-cells. In addition, this triggers the release of cytochrome *c* (Cyt *c*) from the mitochondria, caspase 3 breakage, and apoptosis. Furthermore, TXNIP plays a crucial role in connecting inflammation and ER stress in β-cell death. Elevated glucose levels cause ER stress and upregulate TXNIP expression, which in turn encourages ER stress-induced activation of the NLRP3 inflammasome, generation of IL-1β that results in β-cell death [71].

As TXNIP overexpression in animal studies has been linked to pancreatic β-cells apoptosis [72], TXNIP has been evaluated in the current study. Interestingly, we found a remarkable decline in the expression of TXN2 with a significant upregulation expression of TXNIP, phosphorylated ASK-1, NLRB3and Caspase 3 in the STZ-induced diabetic untreated group. Our finding coincided with previous results which found that glucose stimulates the production of TXNIP in beta cells [73] and documented that TXNIP is an important connection between glucose toxicity and β -cell apoptosis [9,11,74]. Chen J. et al. stated that TXNIP deficiency in insulin (INS-1) cells was capable of avoiding the mitochondrial death cascade in a hyperglycemic state [75] and improving glucose homeostasis. On reviewing the literature, several studies hypothesized the mechanisms by which TXNIP inhibition could be a potential therapeutic strategy in the management of T1DM patients. The studies demonstrated two mechanisms of action either TXNIP inhibitors will inhibit its downstream NLRP3 inflammasome signaling resulting in amelioration of the inflammatory drive with subsequent decreasing ROS and restoration of redox balance. The other mechanism of action is mediated *via* the modulation of glucose entry and breakdown by cells. Wu et al. revealed that TXNIP can modulate the localization of glucose transporter (Glut1). Moreover, Farrell and his colleagues found that TXNIP inhibits glycolysis by downregulating the expression of glycolytic enzymes [76]. Of note, our results in the diabetic-treated groups 3,4,5, and 6 exhibited promising inhibition to the TXNIP/NLRP3 axis especially the triple therapy in group 6. In alignment with our results, Shao et al. [77] reported that GLP-1 agonist can modulate expression of TXNIP expression by promoting its proteasomal degradation resulting in decreasing its downstream signaling. Noteworthy, this is the first study to report the effect of probiotics and BCG on TXNIP signaling in rat models of type 1 diabetes. Hereby, we can hypothesize that the beneficial effects contributed by probiotics and BCG on glucose homeostasis parameters, might be attributed to enhanced glucose entry and cellular breakdown as a result of being potential TXNIP inhibitors.

Notably, when glucose is stimulated, beta cells release islet amyloid polypeptide (IAPP) along with insulin. IAPP is renowned for its capacity to misfold and generate islet amyloid, which also plays a role in the control of glucose. Smaller cell-toxic intermediates allow amyloid fibrils to develop, the deposited amyloid destroys the typical islet architecture. TXNIP was also found to increase the expression level of IAPP which promotes inflammation and beta-cell cytotoxicity in a previous study done by Jing et al. [78]. Additionally, Paulsson et al. [79] speculated that the marked elevated IAPP concentrations may comprise a risk factor for beta cell destruction in T1DM. These findings further supported our reported data in the STZ-diabetic untreated group that demonstrated a significant surge in pancreatic expression of IAPP.

TXNIP and oxidative stress have also been found to boost β-cell apoptosis by elevating the expression of pro-apoptotic miR-200 [80,81]. Our work sheds light on the links between microRNA to beta cell apoptosis evidenced by the significant upregulation in pancreatic miR-200b and the upgrade in BAX/Bcl2 expression. Consistent with our study, Filios and his colleagues found a remarkable upregulation in the expression of caspase-3 and miR-200b in insulin beta cells in addition to a significant elevation in the percentage of TUNEL-positive beta cells. Furthermore, that study demonstrated the potential involvement of miR-200 in beta cell expansion by upregulating the expression of the epithelial marker E-cadherin while inhibiting the epithelial-mesenchymal transition [82].

It is interesting to note that the triple therapy in group 6 was superior to all other diabetic-treated groups in amelioration or even near normalization in most of the aforementioned apoptotic markers, signifying that our proposed combination regimen could be a new promising therapeutic modality and might be considered in further experimental and clinical research to assess and verify these outcomes, especially at the early onset of type 1 diabetes mellites.

## 6. Conclusion

Results of the current study, for the first time, shed light on the therapeutic potential of combining either liraglutide and/or

probiotic with BCG vaccine in promoting beta cell survival through suppression of TXNIP/NLRP3/IL1β Pyroptosis signaling axis, high lightning the implicated role of this axis in upgrading beta cell oxidative stress, inflammation, and apoptosis thus providing a molecular focus for targeted intervention. Noteworthy, the outcomes of this study need to be verified and validated by further experimental studies that might set the stage for future interventional clinical studies to ensure a robust foundation for translating our findings into a new promising therapeutic strategy for type 1 diabetic patients.

## 7. Study limitation

We did not stop the investigated drugs to evaluate the studied parameters after stopping BCG, liraglutide, and probiotics The proposed treatment interventions may not adequately capture the long-term effects or potential problems due to the relatively short duration of the study period to show complete reversibility of full-blown picture of diabetes in human or to halt the autoimmune nature of the disease. In this regard, we recommend further experimental and clinical studies with longer duration to verify our proposed mechanistic pathway and evaluate the safety and effectiveness of these interventions in clinical settings.

## Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Funding

This research did not receive any grant from funding agencies.

## CRediT authorship contribution statement

**Amira Karam Khalifa:** Writing – review & editing, Writing – original draft, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Dina Sayed Abdelrahim:** Writing – review & editing, Writing – original draft. **Dina Mohamed Mekawy:** Writing – review & editing, Investigation. **Reham Mohammad Raafat Hamed:** Writing – review & editing, Writing – original draft. **Wafaa Rabee Mohamed:** Writing – review & editing, Investigation. **Nagwa Mahmoud Ramadan:** Writing – review & editing, Software. **Mostafa Wael:** Software, Methodology, Data curation, Conceptualization. **Rawan Ellackany:** Resources, Conceptualization. **Emad Ali Albadawi:** Writing – review & editing, Investigation. **Walla'a A. Osman:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38932.

## List of abbreviation

| | |
|---|---|
| **ASK-1** | apoptosis signal-regulating kinase 1 |
| **BAX** | BCL2-Associated X Protein |
| **BCG** | bacille Calmette-Guerin |
| **BCL2** | B-cell lymphoma 2 |
| **CDNA** | complementary DNA |
| **GSH** | Glutathione |
| **IAPP** | Islet amyloid polypeptide |
| **IL-1β** | Interleukin-1 beta |
| **LIR** | liraglutide |
| **MDA** | Malondialdehyde |
| **NFκB** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **NLRB3** | nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3 |
| **qPCR** | quantitative polymerase chain reaction |
| **ROS** | Reactive oxygen species |
| **SOD** | **Superoxide dismutase** |
| **STZ** | Streptozotocin |
| **TNFα** | Tumor necrosis factor alpha |
| **TXN2** | Thioredoxin 2 |

**TXNIP** Thioredoxin-interacting protein

## References


[1] G.A. Gregory, et al., Global incidence, prevalence, and mortality of type 1 diabetes in 2021 with projection to 2040: a modelling study, Lancet Diabetes Endocrinol. 10 (10) (2022) 741–760.
[2] P. In't Veld, Insulitis in human type 1 diabetes: the quest for an elusive lesion, Islets 3 (4) (2011) 131–138.
[3] A.D. Sanlioglu, et al., Clinical utility of insulin and insulin analogs, Islets 5 (2) (2013) 67–78.
[4] S. Tsalamandris, et al., The role of inflammation in diabetes: current concepts and future perspectives, Eur. Cardiol. 14 (1) (2019) 50–59.
[5] M.Y. Donath, S.E. Shoelson, Type 2 diabetes as an inflammatory disease, Nat. Rev. Immunol. 11 (2) (2011) 98–107.
[6] Š. Benáková, B. Holendová, L. Plecitá-Hlavatá, Redox homeostasis in pancreatic β-cells: from development to failure, Antioxidants 10 (4) (2021) 526.
[7] D. Wondafrash, A. Nire, At, G.G. Tafere, D.M. Desta, D. Asmerom, K.A. Zewdie, Thioredoxin-interacting protein as a novel potential therapeutic target in diabetes mellitus and its underlying complications, Diabetes, Metab. Syndrome Obes. Targets Ther. 13 (2020) 43–51.
[8] N. Mohammad Alhawiti, et al., TXNIP in metabolic regulation: physiological role and therapeutic outlook, Curr. Drug Targets 18 (9) (2017) 1095–1103.
[9] M. Okamoto, et al., Endogenous hydrogen sulfide protects pancreatic beta-cells from a high-fat diet-induced glucotoxicity and prevents the development of type 2 diabetes, Biochem. Biophys. Res. Commun. 442 (3–4) (2013) 227–233.
[10] H. Parikh, et al., TXNIP regulates peripheral glucose metabolism in humans, PLoS Med. 4 (5) (2007) e158.
[11] J. Chen, et al., Thioredoxin-interacting protein: a critical link between glucose toxicity and β-cell apoptosis, Diabetes 57 (4) (2008) 938–944.
[12] A.A. Mamun, et al., Role of pyroptosis in diabetes and its therapeutic implications, J. Inflamm. Res. 14 (2021) 2187–2206.
[13] D. Carlos, et al., Mitochondrial DNA activates the NLRP3 inflammasome and predisposes to type 1 diabetes in murine model, Front. Immunol. 8 (2017) 187998.
[14] R. Zhou, et al., Thioredoxin-interacting protein links oxidative stress to inflammasome activation, Nat. Immunol. 11 (2) (2010) 136–140.
[15] M.C. Rousseau, M.E. Parent, Y. St-Pierre, Potential health effects from non-specific stimulation of the immune function in early age: the example of BCG vaccination, Pediatr. Allergy Immunol. 19 (5) (2008) 438–448.
[16] L. Groele, H. Szajewska, A. Szypowska, Effects of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in children with newly diagnosed type 1 diabetes: protocol of a randomised controlled trial, BMJ Open 7 (10) (2017) e017178.
[17] I. Moreno-Indias, F. Tinahones, Probiotics for Diabetes Mellitus: Prevention and Treatment, 2022, pp. 485–502.
[18] D. Yang, et al., Interactions between gut microbes and NLRP3 inflammasome in the gut-brain axis, Comput. Struct. Biotechnol. J. 21 (2023) 2215–2227.
[19] M. von Herrath, et al., Anti-interleukin-21 antibody and liraglutide for the preservation of β-cell function in adults with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled, phase 2 trial, Lancet Diabetes Endocrinol. 9 (4) (2021) 212–224.
[20] W. Zhou, et al., Glucagon-like peptide-1 receptor mediates the beneficial effect of liraglutide in an acute lung injury mouse model involving the thioredoxin-interacting protein, Am. J. Physiol. Endocrinol. Metabol. 319 (3) (2020) E568–E578.
[21] A. Chen, et al., Liraglutide attenuates NLRP3 inflammasome-dependent pyroptosis via regulating SIRT1/NOX4/ROS pathway in H9c2 cells, Biochem. Biophys. Res. Commun. 499 (2) (2018) 267–272.
[22] H. Ao, et al., TXNIP positively regulates the autophagy and apoptosis in the rat müller cell of diabetic retinopathy, Life Sci. 267 (2021) 118988.
[23] Y. Wang-Fischer, T. Garyantes, Improving the reliability and utility of streptozotocin-induced rat diabetic model, J. Diabetes Res. 2018 (2018) 8054073.
[24] M. Sariyanti, et al., Probiotic Lactobacillus acidophilus FNCC 0051 improves pancreatic histopathology in streptozotocin-induced type-1 diabetes mellitus rats, The Indonesian Biomedical Journal 14 (4) (2022) 410–415.
[25] B.L. Furman, Streptozotocin-induced diabetic models in mice and rats, Curr. Protoc. Pharmacol. 70 (2015) 5.47.1–5.47.20.
[26] Y. Dong, et al., Studies on the antidiabetic activities of Cordyceps militaris extract in diet-streptozotocin-induced diabetic Sprague-Dawley rats, BioMed Res. Int. 2014 (2014) 160980.
[27] C. Palleria, et al., Liraglutide prevents cognitive decline in a rat model of streptozotocin-induced diabetes independently from its peripheral metabolic effects, Behav. Brain Res. 321 (2017) 157–169.
[28] S.M. Man, T.-D. Kanneganti, Regulation of inflammasome activation, Immunol. Rev. 265 (1) (2015) 6–21.
[29] K. Hong, et al., Cytokines regulate β-cell thioredoxin-interacting protein (TXNIP) via distinct mechanisms and pathways, J. Biol. Chem. 291 (16) (2016) 8428–8439.
[30] J. Su, et al., Research progress on drugs for diabetes based on insulin receptor/insulin receptor substrate, Biochem. Pharmacol. (2023) 115830.
[31] F. Teufel, et al., Body-mass index and diabetes risk in 57 low-income and middle-income countries: a cross-sectional study of nationally representative, individual-level data in 685 616 adults, Lancet 398 (10296) (2021) 238–248.
[32] P. Choudhary, et al., Evidence-informed clinical practice recommendations for treatment of type 1 diabetes complicated by problematic hypoglycemia, Diabetes Care 38 (6) (2015) 1016–1029.
[33] A. Abu-Zaid, et al., A systematic review and dose-response meta-analysis on the efficacy of dapagliflozin in patients with type 1 diabetes mellitus, Pharmacol. Res. 165 (2021) 105456.
[34] A. Llano, G.A. McKay, Non-insulin therapies in addition to insulin in Type 1 DM treatment, Br. Med. Bull. 134 (1) (2020) 54–62.
[35] P. Leete, et al., Differential insulitic profiles determine the extent of β-cell destruction and the age at onset of type 1 diabetes, Diabetes 65 (5) (2016) 1362–1369.
[36] S.A. Tersey, et al., Islet β-cell endoplasmic reticulum stress precedes the onset of type 1 diabetes in the nonobese diabetic mouse model, Diabetes 61 (4) (2012) 818–827.
[37] H. Huang, Q. Wang, J. Buteau, A novel small-molecule activator of lyn kinase for the treatment of type 1 diabetes, Can. J. Diabetes 46 (7) (2022) S30.
[38] B.L. Furman, Streptozotocin-induced diabetic models in mice and rats, Curr Protoc 1 (4) (2021) e78.
[39] B.T. Zhu, Pathogenic mechanism of autoimmune diabetes mellitus in humans: potential role of streptozotocin-induced selective autoimmunity against human islet β-cells, Cells 11 (3) (2022) 492.
[40] J. Wu, L.-J. Yan, Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic β cell glucotoxicity, Diabetes, Metab. Syndrome Obes. Targets Ther. (2015) 181–188.
[41] E. Faruk, Pancreatic protection elicited by platelet-rich plasma and cinnamon combination in a rat model of type 1 diabetes: is it a new era in islet cell regeneration and insulin signalling genes? Egyptian Journal of Histology 45 (3) (2022) 720–737.
[42] W. Bielka, A. Przezak, A. Pawlik, The role of the gut microbiota in the pathogenesis of diabetes, Int. J. Mol. Sci. 23 (1) (2022) 480.
[43] M.C. de Goffau, et al., Aberrant gut microbiota composition at the onset of type 1 diabetes in young children, Diabetologia 57 (2014) 1569–1577.
[44] H. Siljander, J. Honkanen, M. Knip, Microbiome and type 1 diabetes, EBioMedicine 46 (2019) 512–521.
[45] L.A. Velloso, F. Folli, M.J. Saad, TLR4 at the crossroads of nutrients, gut microbiota, and metabolic inflammation, Endocr. Rev. 36 (3) (2015) 245–271.
[46] A. Abdelazez, et al., Screening potential probiotic characteristics of Lactobacillus brevis strains in vitro and intervention effect on type I diabetes in vivo, BioMed Res. Int. (2018), 2018.
[47] A.R. Moravejolahkami, et al., Probiotics, prebiotics, and synbiotics in type 1 diabetes mellitus: a systematic review and meta-analysis of clinical trials, Diabetes Metab Res Rev 40 (2) (2024) e3655.
[48] S.H. Baïk, et al., BCG vaccine prevents insulitis in low dose streptozotocin-induced diabetic mice, Diabetes Res. Clin. Pract. 46 (2) (1999) 91–97.
[49] M. Karaci, Chapter 4-the protective effect of the BCG vaccine on the development of type 1 diabetes in humans. The Value of BCG and TNF in Autoimmunity, Academic Press, Amsterdam, 2014 [Internet].

[50] M. Karaci, The protective effect of the BCG vaccine on the development of type 1 diabetes in humans, in: The Value of BCG and TNF in Autoimmunity, Elsevier, 2014, pp. 52–62.
[51] M.E. Doyle, J.M. Egan, Mechanisms of action of glucagon-like peptide 1 in the pancreas, Pharmacol. Therapeut. 113 (3) (2007) 546–593.
[52] A. Villalba, et al., Repurposed analog of GLP-1 ameliorates hyperglycemia in type 1 diabetic mice through pancreatic cell reprogramming, Front. Endocrinol. 11 (2020) 258.
[53] A. Alnahdi, A. John, H. Raza, N-acetyl cysteine attenuates oxidative stress and glutathione-dependent redox imbalance caused by high glucose/high palmitic acid treatment in pancreatic Rin-5F cells, PLoS One 14 (12) (2019) e0226696.
[54] S.M. El-Megharbel, et al., Synthesis of new antidiabetic agent by complexity between vanadyl (II) sulfate and vitamin B1: structural, characterization, anti-DNA damage, structural alterations and antioxidative damage studies, Appl. Organomet. Chem. 33 (5) (2019) e4892.
[55] I.C. West, Radicals and oxidative stress in diabetes, Diabet. Med. 17 (3) (2000) 171–180.
[56] A. Piwkowska, et al., High glucose concentration affects the oxidant-antioxidant balance in cultured mouse podocytes, J. Cell. Biochem. 112 (6) (2011) 1661–1672.
[57] M.S. Refat, et al., Quercetin/Zinc complex and stem cells: a new drug therapy to ameliorate glycometabolic control and pulmonary dysfunction in diabetes mellitus: structural characterization and genetic studies, PLoS One 16 (3) (2021) e0246265.
[58] H. Kim, et al., Antioxidant and probiotic properties of Lactobacilli and Bifidobacteria of human origins, Biotechnol. Bioproc. Eng. 25 (3) (2020) 421–430.
[59] W.I. Izuddin, et al., Dietary postbiotic Lactobacillus plantarum improves serum and ruminal antioxidant activity and upregulates hepatic antioxidant enzymes and ruminal barrier function in post-weaning lambs, Antioxidants 9 (3) (2020) 250.
[60] L. Qi, et al., Liraglutide reduces oxidative stress and improves energy metabolism in methylglyoxal-induced SH-SY5Y cells, Neurotoxicology 92 (2022) 166–179.
[61] M. El-Shafey, et al., Role of dapagliflozin and liraglutide on diabetes-induced cardiomyopathy in rats: implication of oxidative stress, inflammation, and apoptosis, Front. Endocrinol. 13 (2022).
[62] W.S. Elseady, et al., New insight on the role of liraglutide in alleviating dexamethasone-induced pancreatic cytotoxicity via improving redox status, autophagy flux, and PI3K/Akt/Nrf2 signaling, Can. J. Physiol. Pharmacol. 99 (11) (2021) 1217–1225.
[63] X. Li, et al., Activation of NF-κB-inducing kinase in islet β cells causes β cell failure and diabetes, Mol. Ther. 28 (11) (2020) 2430–2441.
[64] D.L. Eizirik, et al., The human pancreatic islet transcriptome: expression of candidate genes for type 1 diabetes and the impact of pro-inflammatory cytokines, PLoS Genet. 8 (3) (2012) e1002552.
[65] Y. Zhang, et al., Lactobacillus Plantarum NC8 and its metabolite acetate alleviate type 1 diabetes via inhibiting NLRP3, Microb. Pathog. 182 (2023) 106237.
[66] X. Chen, et al., GLP-1 alleviates NLRP3 inflammasome-dependent inflammation in perivascular adipose tissue by inhibiting the NF-κB signalling pathway, J. Int. Med. Res. 49 (2) (2021) 0300060521992981.
[67] A. Villalba, et al., Repurposed analog of GLP-1 ameliorates hyperglycemia in type 1 diabetic mice through pancreatic cell reprogramming, Front. Endocrinol. 11 (2020) 258.
[68] S. Mishra, et al., Probiotics and prebiotics for the amelioration of type 1 diabetes: present and future perspectives, Microorganisms 7 (3) (2019) 67.
[69] Y. Zhang, et al., Lactobacillus Plantarum NC8 and its metabolite acetate alleviate type 1 diabetes via inhibiting NLRP3, Microb. Pathog. 182 (2023) 106237.
[70] S.-H. Wei, Y.-P. Chen, M.-J. Chen, Selecting probiotics with the abilities of enhancing GLP-1 to mitigate the progression of type 1 diabetes in vitro and in vivo, J. Funct.Foods 18 (2015) 473–486.
[71] E.-H. Choi, S.-J. Park, TXNIP: a key protein in the cellular stress response pathway and a potential therapeutic target, Exp. Mol. Med. 55 (7) (2023) 1348–1356.
[72] N.M. Alhawiti, et al., TXNIP in metabolic regulation: physiological role and therapeutic outlook, Curr. Drug Targets 18 (9) (2017) 1095–1103.
[73] A.H. Minn, C. Hafele, A. Shalev, Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces β-cell apoptosis, Endocrinology 146 (5) (2005) 2397–2405.
[74] J. Lu, A. Holmgren, Thioredoxin system in cell death progression, Antioxidants Redox Signal. 17 (12) (2012) 1738–1747.
[75] J. Chen, et al., Lack of TXNIP protects against mitochondria-mediated apoptosis but not against fatty acid–induced ER stress–mediated β-cell death, Diabetes 59 (2) (2010) 440–447.
[76] M.R. Farrell, et al., Thioredoxin-interacting protein inhibits hypoxia-inducible factor transcriptional activity, Free Radic. Biol. Med. 49 (9) (2010) 1361–1367.
[77] W. Shao, et al., Cyclic AMP signaling stimulates proteasome degradation of thioredoxin interacting protein (TxNIP) in pancreatic β-cells, Cell. Signal. 22 (8) (2010) 1240–1246.
[78] G. Jing, et al., Thioredoxin-interacting protein promotes islet amyloid polypeptide expression through miR-124a and FoxA2, J. Biol. Chem. 289 (17) (2014) 11807–11815.
[79] J.F. Paulsson, et al., High plasma levels of islet amyloid polypeptide in young with new-onset of type 1 diabetes mellitus, PLoS One 9 (3) (2014) e93053.
[80] A. Shalev, Minireview: thioredoxin-interacting protein: regulation and function in the pancreatic β-cell, Mol. Endocrinol. 28 (8) (2014) 1211–1220.
[81] A. Magenta, et al., miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition, Cell Death Differ. 18 (10) (2011) 1628–1639.
[82] S.R. Filios, et al., MicroRNA-200 is induced by thioredoxin-interacting protein and regulates Zeb1 protein signaling and beta cell apoptosis, J. Biol. Chem. 289 (52) (2014) 36275–36283.